April 2012

Human Ependymin Neurotrophic Factor Mimetic Increases Alzheimer's Mice Cognitive Performance in a Morris Water-Maze Test

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MQP-BIO-DSA-2454
MQP-ID-DSA-7647

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A Major Qualifying Project Report

Submitted to the Faculty of the

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the

Degrees of Bachelor of Science

in

Biology and Biotechnology (TJ and MN)

and in

Biochemistry (MN)

by

_____ _____

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April 26, 2012

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ABSTRACT

Alzheimer's disease (AD) is a neurodegenerative disease thought to be caused by the abnormal production and/or low clearance of neurotoxic amyloid-beta. Using transgenic mice that mimic AD, this project measured whether AD mice treated with a neurotrophic factor mimetic previously shown to increase human neuronal survival *in vitro* improves mouse behavior. A Morris water-maze swimming test that requires a functional hippocampus was used to compare learning behavior in WT and AD mice. AD mice took on average 25 seconds to locate the hidden platform on the final day of testing, while WT located the platform in roughly 10 seconds ($p = 0.05$). Treated AD mice showed a 19% decrease ($p < 0.01$) relative to untreated AD mice in the distance traveled to locate a marked platform, and a 24% decrease in the distance traveled to locate an unmarked platform.

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ACKNOWLEDGEMENTS

First and foremost we would like to send our sincerest thanks to our project advisor, Professor Dave Adams, for his continuous support and guidance throughout this project. His insight and expertise into the field of Alzheimer's, as well as his genuine interest in the topic, served as an inspiration to us both. His vast knowledge and experience served a pivotal role in our ability to complete and troubleshoot through our preliminary investigations. He has taught us to think critically and logically, thus making us better scientists and preparing us to become future leaders in our field.

We would also like to thank PsychoGenics (Tarrytown, NY) for conducting the Morris water maze tests, and providing mouse brain and tail samples, as well as monetary support for the completion of this project. Lastly, we wish to thank former students Melanie Donahue and Gregory Lobdell for providing the original SHSY-5Y cell stocks used in our *in vitro* studies.

BACKGROUND

Alzheimer's Disease

Alzheimer's disease (AD) is the most common form of dementia in the U.S., causing deficiencies in logic, memory, and behavior. Its prevalence has been increasing, with 13% of Americans over the age of 65 currently diagnosed with AD, making it the fifth leading cause of death among that age group (Alzheimer's Facts, 2010). AD was first characterized in 1901 in a 51 year-old woman (Auguste Deter) who complained of a lost self-identity. She was cared for by a German physician, Dr. Alois Alzheimer, who characterized her dementia, and later in 1906 for the brain autopsy showed the existence of the main hallmark cellular features of the disease: extracellular senile plaques and intracellular neurofibrillary tangles (Alzheimer's Foundation of America, 2010). Up until now, a post-mortem brain autopsy was the most conclusive method of diagnosing the disease (Progress Report, 2009; Alzheimer's Foundation of America, 2010).

AD Symptoms

AD symptoms tend to worsen with age. Early on, patients experience impaired memory such as problems recalling names, recent activities, placement of objects, as well as a general disorientation. Other warning signs include withdrawal from social events, changes in personality, irritability, and apathy (Progress Report, 2009; Alzheimer's Facts, 2010). As the disease progresses, there is a further decline in cognitive and functional abilities. Patients begin to struggle with speaking, swallowing, making judgments, or recognizing family members or loved ones (Alzheimer's Foundation of America, 2010). Difficulty with easy motor tasks, such as using a spoon or putting on clothes, becomes more evident. Some patients who develop psychotic symptoms experience auditory and/or visual hallucinations, and sensory impairment

(Progress Report, 2009; Alzheimer's Foundation of America, 2010). Symptoms typically begin to appear between the ages of 60–70, but can appear as early as the 40's for early on-set cases. Once diagnosed, patients typically live another 8-10 years. Death usually results from pneumonia resulting from complications of being immobile.

AD Cellular Pathology

At the cellular level, specific neurons that make up the memory control centers of the brain (the entorhinal cortex and the hippocampus) begin to lose their connections and die due to disruption of communication, metabolism, and repair (Progress Report, 2009). Major damage is observed in the temporal region, and with further progression the cortex (the brain region responsible for communication and reasoning) also becomes damaged (**Figure-1**).

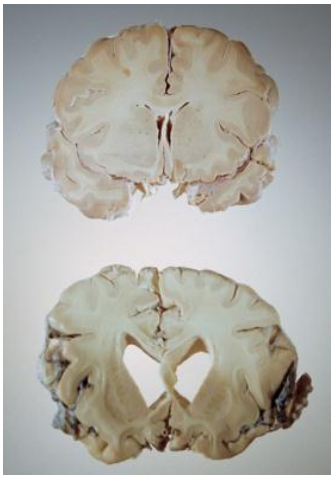


Figure-1: Photograph of a Normal Human Brain (upper) and an AD Brain (lower). Note the appearance of areas of the AD brain with loss of gray and white matter. ((Sanders, 2011)

The two hallmark pathological features of AD are: 1) intracellular neurofibrillary tangles (NFTs), interwoven fibers caused by abnormally hyper-phosphorylated tau proteins, 2) extracellular senile plaques, aggregations of over-expressed beta-amyloid (Aβ) protein, and 3)

congophilic angiopathy, the formation of amyloid depositions in the blood vessel walls of the central nervous system (Progress Report, 2009).

AD Diagnosis

In the case of a progressive, irreversible disease, it is vital that an early diagnosis is made so that the affected individuals can make plans while they still have cognitive function. Until now, there has not been a conclusive method of AD diagnosis besides the standard post-mortem brain autopsy (Progress Report, 2009; Alzheimer's Foundation of America, 2010). However, clinicians now use a combination of assessments (**Figure-2**).

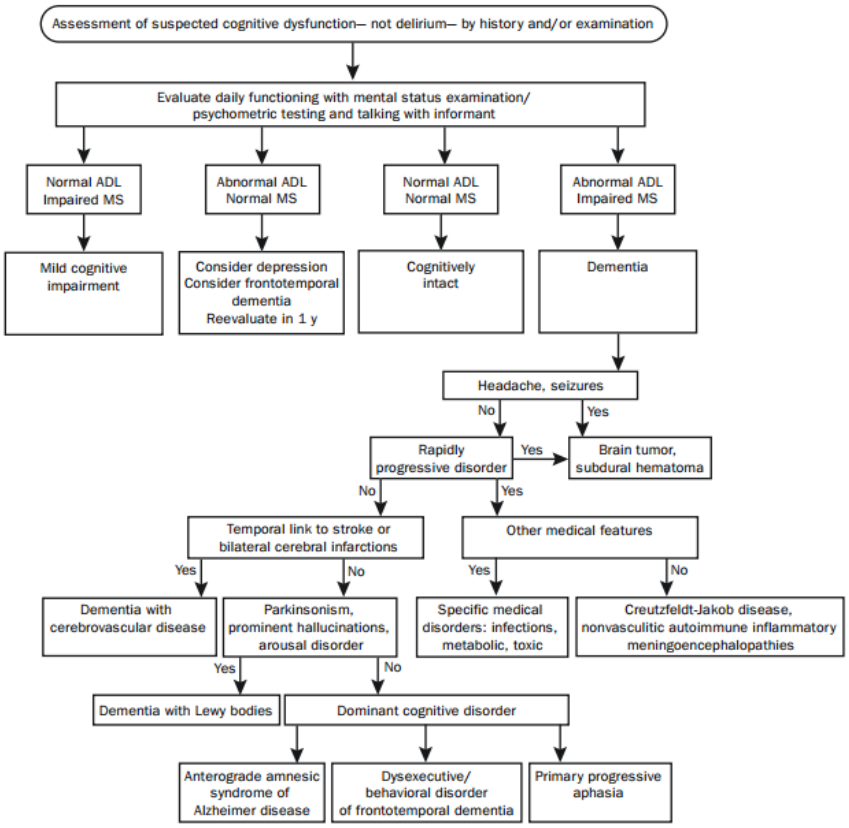


Figure-2: Diagnosis Scheme for Mental Dysfunction. The assessment begins with information gathered from previous family history or talking to the patient, with special attention to any dependence they have on others for their activities of daily living (ADL), an indicator of mental status (MS). Medical conditions such as seizures, hallucinations, and risk of stroke are also important features in the diagnosis (Knopman et al., 2003).

Mental assessment typically begins by studying patient and family medical histories, as well as periodic physical examinations to assess a patient's level of memory and language ability. Although it not useful for distinguishing AD from other forms of dementia, the Mini Mental State Examination (MMSE) has been the most prevalent screening test for dementia due to its ease and rapidity (Progress Report, 2009). Unfortunately, these assessments neglect to account for mild changes in cognition, but instead diagnose moderate to severe AD. Research has indicated that AD begins to invade the brain long before clinical symptoms manifest. Thus, much research has focused on developing sophisticated protocols to detect early AD.

The written MMSE has now been remodeled into a number of computer-based examinations with automated scoring (Saxton *et al*., 2009). A collaborative effort of various US academic research groups, known as the Alzheimer's Disease Neuroimaging Initiative (ADNI), is performing multiple longitudinal studies to identify an AD biomarker only observed in the CSF of AD patients (Progress Report, 2009). Biomarker levels are being assessed using multiple brain imaging technologies, including PET amyloid imaging, and diffusion, functional, and spectroscopic MRIs. PET imaging with florbetapir has been shown to identify up to 96% of AD cases as later confirmed by post-mortem brain autopsies (Clark *et al*., 2011). In addition, the plasma levels of Aβ have been shown to decrease with AD, presumably in response to its sequestration in the brain as the senile plaques form, potentially providing an AD blood test (Yaffe *et al*., 2011).

AD Risk Factors

The largest non-hereditary risk factor of AD is advancing age. Due to reasons not fully understood, upon reaching the age of 65 the chances of developing AD doubles every 5 years,

reaching nearly 50% by the age of 85 (Alzheimer's Facts, 2010). The average age of the U.S. population is increasing, and the average number of people living over 100 years is expected to be 15 times greater in 50 years (Progress Report, 2009). Research suggests that as neurons age, there is increased mitochondrial fission, a larger reliance to fats and amino acids (instead of the usual glucose) for energy production, and an increase in insulin-like growth-factor-1 (IGF-1), a hormone responsible for cell growth (Yao *et al*., 2009; Kadish *et al*., 2009). Each of these cellular ageing effects also boosts Aβ production.

Genetic mutations have also been shown to increase the production of toxic Aβ as a result of higher amyloid precursor protein (APP) processing. Early-onset familial AD (FAD) has been strongly linked with the following three gene mutations: amyloid precursor protein (APP) on chromosome-21, presenilin-1 (PS-1) on chromosome-14, and presenilin-2 (PS-2) on chromosome-1 (Progress Report, 2009; Alzheimer's Facts, 2010). A fourth gene mutation known to increase AD risk is Apolipoprotein E (ApoE) on chromosome-19. Other factors implicated to increase AD risk include head traumas and cardiovascular disease.

AD Prevalence

Accounting for roughly 60-80% of all dementia cases in 2011, AD was classified as the most common type of dementia in 2011. Approximately 5.4 million Americans currently have AD. Of this number, 45% are 85 and older, 45% are age 75-84, and 6% are 65-74 years of age. A significant 13% (1 in 8) Americans aged 65 and older have AD (Herbert *et al.* 2003). Globally, Alzheimer's Disease International (ADI) estimates that 30 million people had AD in 2008, and a projected 100 million will be affected by 2050 (Alzheimer's Disease International, 2010). Annually, 4.6 million new cases of AD (one new case every seven seconds) are diagnosed

(Alzheimer's Facts, 2011). Outside the U.S., the trend of increasing AD prevalence with increased age has also been verified for Europe, the Middle East/North Africa, Asia, and Sub-Saharan Africa. For all populations (**Figure-3**), AD is predicted to increase in prevalence more rapidly in low to middle income countries (red), than high income nations (brown).

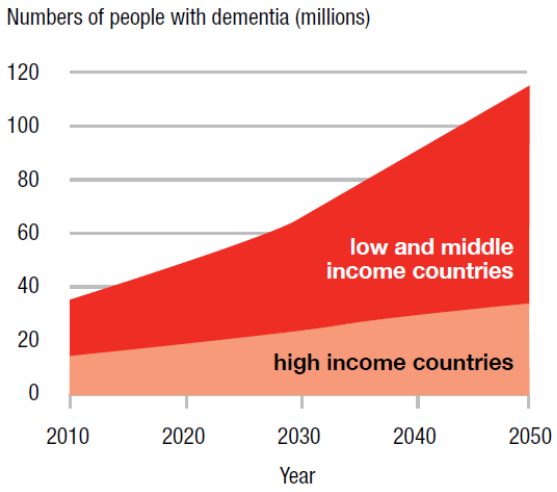


Figure- 3: Estimates for AD Prevalence According to Income. The number of individuals affected by AD dementia in low and middle income countries (red) is more than twice that of high income countries (brown). By 2050 the low and middle income country populations affected by AD will be three times those affected in high income countries. (Alzheimer's Disease International, 2010).

Almost two-thirds of all Americans living with the disease are women. Of those living with Alzheimer's over the age of 65, 3.4 million are women and 1.8 million are men (Herbert *et al.*, 2003; Shriver, 2010). Further research showed that the difference in proportion is likely due to the higher average life expectancy of women. When incidence was measured using age-specific criteria, a significant difference between genders did not exist (Bachman *et al.* 1993, Fillenbaum *et al.*, 1998; Fitzpatrick *et al.*, 2004; Herbert *et al.*, 2001; Kukull *et al.*, 2002).

Researchers have also studied the difference in incidence of AD among ethnic groups in the U.S. It was found that the majority of people living with AD are non-Hispanic whites.

However, above the age of 75, African-Americans and Hispanics are, respectively, 2.0 and 1.5 times more likely to get AD (**Figure-4**) (Gurland *et al.* 1999; Potter *et al.*, 2009). This difference can potentially be attributed to disparities in socioeconomic backgrounds, such as lower education levels, increased incidence of diabetes and high blood pressure, or other characteristics.

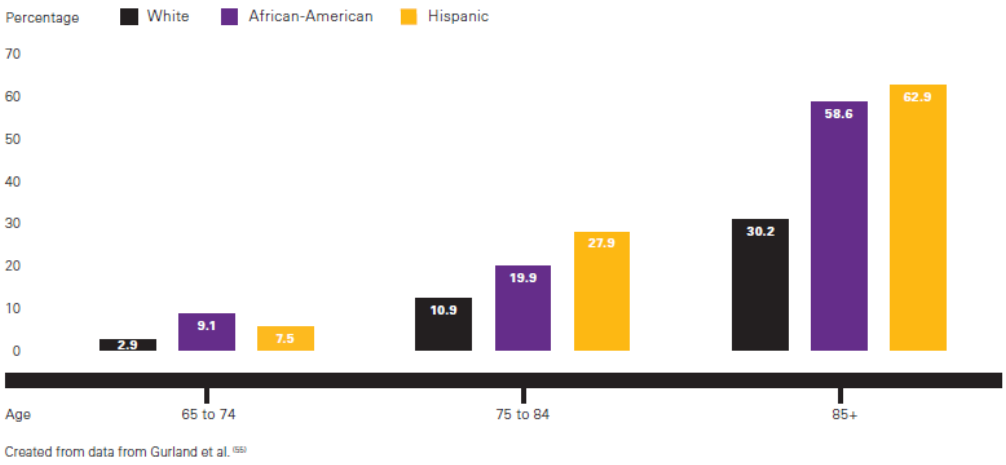


Figure-4: AD Prevalence by Race. Shown are the proportions of people aged 65 and older with Alzheimer's disease and other dementias by race/ethnicity. (Alzheimer's Facts & Figures, 2011)

The level of education is also associated with AD prevalence. It was found that higher years of education correlate with a decreased probability of developing dementia (Stern *et al.*, 1994; Evans *et al.*, 1997; Gurland *et al.*, 1999; Kulkull *et al.* 2002; Evans *et al.*, 2003; Fitzpatrick *et al.*, 2004; Plassman *et al.*, 2007). Researchers speculate that additional education may allow for the development of a "cognitive reserve," which enables individuals to better cope with the physiological effects of AD (Roe *et al.*, 2007; Stern *et al.*, 2006). It is also possible that this difference may simply be due to an overall greater risk for disease and limited medical care for lower socioeconomic groups (McDowell *et al.*, 2007).

As the average life expectancy continues to increase due to advances in medicine and the projected betterment of social and environmental conditions, the incidence of Alzheimer's is also expected to increase. By the year 2030, the U.S. population above 65 years is predicted to double. These estimated 71 million older Americans will make up roughly 20 percent of the total population. As depicted in **Figure-5**, with this increase in the number of older Americans, the number of individuals affected by AD will reach roughly 13.2 million by the year 2050.

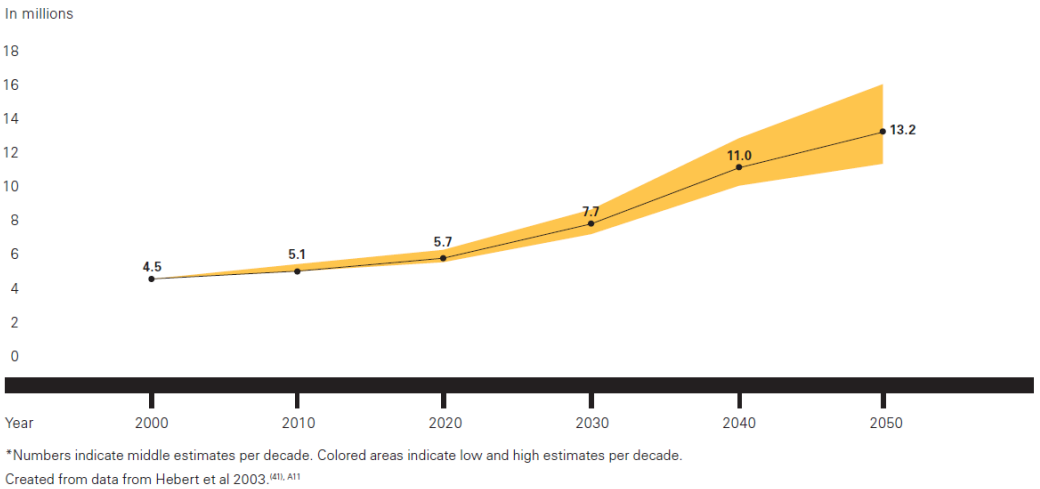


Figure-5: Projected Incidence of Alzheimer's Disease from 2000-2050. The number of individuals in the U.S. diagnosed with AD is projected to reach 13.2 million by 2050. (Alzheimer's Facts & Figures, 2011)

AD Therapies

Currently, there are no treatments available to stop or reduce brain deterioration in AD. However, five drugs have been approved by the U.S. Food and Drug Administration (FDA) which temporarily slow the worsening of symptoms. These drugs are effective in about half the individuals who use them, and remain effective for only about 6-12 months. The 5 approved drugs fall into two classes: 1) those that inhibit acetylcholinesterase, decreasing the degradation of acetylcholine in the synapse increasing its levels there, and 2) inhibitors of NMDA (N-methyl-

D-aspartate) receptor, which reduce the levels of excitotoxic glutamate in the synapse increasing memory and learning. The acetylcholinesterase inhibitors include *Donepezil* (Aricept, treats all stages), *Galantamine* (Razadyne, treats mild to moderate AD), *Rivastigmine* (Exelon, mild to moderate AD) and *Tacrine* (Cognex, mild to moderate AD). The remaining FDA approved drug, *Memantine* (Namenda), is the only NMDA inhibitor, and is used to treat moderate to severe AD. As a glutamate regulator, *Memantine* works by partially blocking NMDA receptors on the cell surface, thus preventing excitotoxic glutamate from binding the receptor to induce cell damage (Alzheimer's Association, 2012). Several non-drug therapies have also been developed which aid in limiting memory loss, and correcting behavioral and sleep changes. Still, all current therapies work to mask AD symptoms rather than treat the underlying disease.

Several national and international research associations are dedicated to finding an AD cure, including the NIA Division of Neuroscience Translational Research Platform and Alzheimer's Disease Centers, the Genetics of Alzheimer's Disease Data Storage Site, the National Alzheimer's Coordinating Center, and the National Cell Repository for Alzheimer's Disease (Alzheimer's Association, 2012). Much of the current research focuses on trying to treat the disease mechanism, not just the symptoms. Drugs have been created to alter major AD targets such as tau protein, Aβ, insulin resistance, and inflammation. Other treatments approved for non-Alzheimer's disorders, such as nonsteroidal anti-inflammatory drugs (NSAIDS), anti-depressants, immune-modulatory polypeptide glatiramer (multiple sclerosis treatment), sildenafil (Viagra®), and antipsychotics, have also been tested. A recent study showed that the cancer drug exarotene (Targretin) was capable of improving memory and reversing signs of Alzheimer's in mouse brains within 72 hours. Bexarotene was approved by the FDA in 1999 for treating cutaneous T-cell lymphoma, however research later found that it also turns on a gene that makes

ApoE, a naturally occurring neuronal protein that facilitates the proteolytic degradation and removal of toxic Aβ, so Bexarotene may also have AD applications (Cramer *et al.*, 2012; Jiang *et al.*, 2008).

Though the search continues, researchers predict that science may soon find a solution, and that patients may soon require a drug cocktail that targeting several processes to eliminate the disease. Cocktails will likely include drugs that block the synthesis of new Aβ (i.e. Merck's gamma-secretase inhibitor), drugs that remove existing Aβ (i.e. Elan Pharmaceuticals Aβ vaccines), and drugs that regrow neurons (i.e. using neuronal stem cells or neurotrophic factors, see below). Among the numerous studies occurring, an estimated 75-100% are entering human clinical trials.

Socioeconomic Toll

As the number of individuals affected by AD continues to grow, the projected socioeconomic toll will continue to rise. Although medical care has improved over the last 8 years, and death due to many diseases has decreased, death caused by AD has increased by more than 66% (**Figure-6**), leaving AD as the fifth leading cause of death for those aged 65 or older in the U.S.

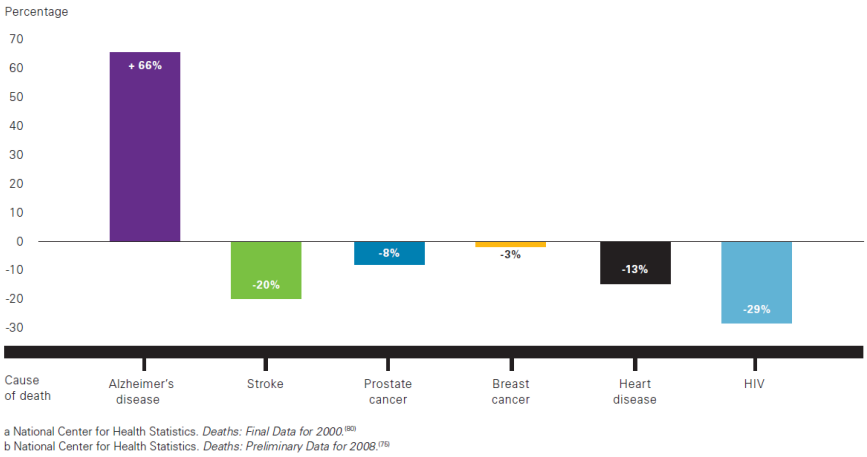


Figure-6: Changes in Causes of Death for All Ages from 2000-2008(All Ages). Note the 66% increase in AD (purple) compared to the decline in stroke, prostate cancer, breast cancer, heart disease, and HIV. (Alzheimer's Facts, 2010)

As stated by Harry John, President and CEO of the Alzheimer's Association, "The scale of the Alzheimer's public health crisis is clear. As many as 5.4 million Americans are living with Alzheimer's today, with an economic toll of $183 billion per year" (Alzheimer's Association, 2012). In 2010, globally the economic cost of dementia was an estimated $604 billion (USD) per year. This value includes both paid and unpaid care, as well as the direct costs of social and medical care. Of this value, medical care makes up a mere 16 percent. Although roughly 15 million Americans provide unpaid care for AD individuals, the cost of AD healthcare, hospice, and long-term care is expected to increase to $1.1 trillion by 2050.

Alzheimer's Cell Death Mechanism, Amyloid Cascade Hypothesis

At the cellular level, AD is characterized by severe neuronal loss and degradation primarily within the outer layers of the cerebral cortex. The temporal and frontal lobes are the first to be affected, followed by the parietal and occipital lobes. During this time of neuronal

loss, other cellular AD hallmarks also develop, including the formation of extracellular Aβ senile plaques, the formation of intracellular tau neurofibrillary tangles (NFTs), and synaptic and neuronal loss (Selkoe, 2001; Masters *et al.*, 2006). These hallmarks provide a premise for the most widely accepted hypothesis for AD pathogenesis, the Amyloid Cascade Hypothesis (ACH). According to the ACH, AD begins with the extracellular formation and eventual deposition of neurotoxic amyloid beta (Aβ), a 40-42 amino acid peptide derived from the proteolytic cleavage of amyloid precursor protein (APP) (Cappai & White, 1999; Selkoe, 2001; Masters *et al.*, 2006; Crews and Masliah, 2010). The senile plaques formed often aggregate in regions involved in memory and cognition, and have been implicated in the disruption of long term potentiation, the accumulation of hyper-phosphorylated non-functional tau protein, and the generation of cellular reactive oxidative stress (ROS) (Butterfield, 2006). Aβ's toxic effects are largely due to its interaction with the 'receptor for advance glycation end products' (RAGE) (Yan *et al.*, 1996). Aβ-RAGE interactions cause ROS in endothelial, microglial, and neuronal cells, and the activation of microglia. Researchers have also found that RAGE expression is elevated in AD patients (Karran *et al.*, 2011; Yan *et al.*, 1996), creating a positive feedback loop with Aβ.

The main tenant of the amyloid cascade hypothesis is that Aβ *initiates* the disease, and then the ROS and tau damage it creates spreads throughout the brain (**Figure-7**). Transgenic mice lacking Aβ but containing tau do not get AD, and transgenic mice containing Aβ but missing tau also do not get AD (Rapoport *et al.*, 2002; Robertson *et al.*, 2007). It is not clear how Aβ formation leads to the formation of neurofibrillary tangles (NTF), however transgenic mouse experiments, where mice are engineered to mimic human early-onset FAD mutations in APP or PSEN1 (gamma secretase), show that the mutations cause an increase in Aβ formation and tau hyper-phosphorylation. Under normal conditions, tau protein functions to stabilize

microtubules, however it can self-assemble in certain neuronal disorders (Avila *et al.*, 2004). Hyper-phosphorylated tau no longer acts to stabilize microtubules, so microtubules disassemble and the neuronal axons shorten, weakening the synapses (Lovestone & Reynolds 1997). NTFs are also present in several other neurodegenerative disorders, including Parkinson's and Hallervorden-Spatz disease (Perl, 2000).

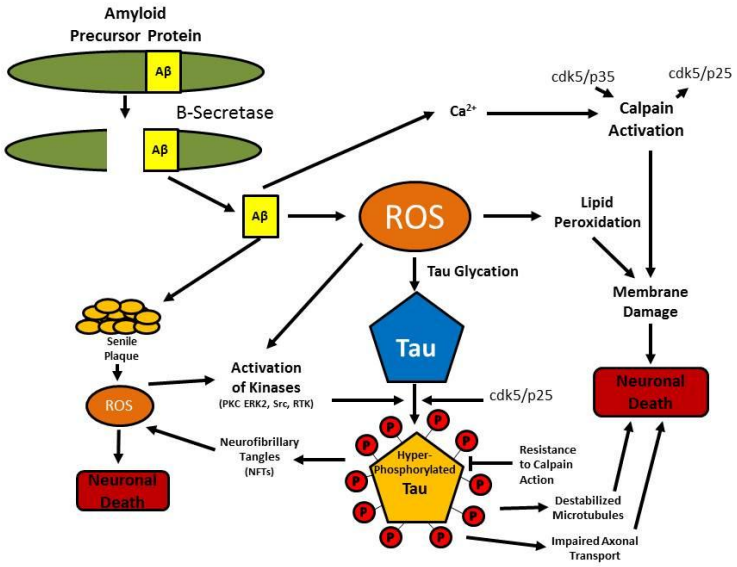


Figure-7: Diagram of the Amyloid Cascade Hypothesis for Alzheimer's Disease. Note that the formation of Aβ (yellow, upper left) from the amyloid precursor protein (green, upper left) initiates the formation of cellular oxidative stress (ROS, orange). Tau hyper-phosphorylation (red) leads to the disassembly of axonal microtubules, weakening the synapse. (*Derived from Adams Review.)*

Although a great deal of evidence supports the role of Aβ and tau in AD, how the damage spreads to other cells was unknown until this year. Two main hypotheses exist for the spread of the neurodegeneration: 1) the disease spreads from neuron-to-neuron via methods used for normal cell communication, or 2) the brain is more resistant to degeneration in some areas than others. Recent evidence supports the former hypothesis. In a study involving mice that expressed hyper-phosphorylated human tau only in their entorhinal cortexes, researchers

observed the slow spread of NTFs to other regions of the brain over two years. The likely explanation for the spread of disease is through neuron-to-neuron interactions (Liu *et al.*, 2012).

Alzheimer's Mouse Behavioral Tests

Since the initial 1995 discovery of the AD mouse model carrying the Indiana FAD mutation in APP under the control of a PDGF promoter causing Aβ expression in the hippocampus and cortex (Games *et al*., 1995), other transgenic mice have been created carrying up to five different APP/PSEN mutations that mimic the disease. These models have been extensively used to study AD progression and to test various therapies, and have been at the forefront of scientific research. These mice make suitable models because they show Aβ formation in the same regions of the brain as AD patients and initiate neurodegeneration. Like other mice, they are hardy, adaptable, and are small in size, which allows for easy housing and relatively large trials.

Interestingly, the AD mouse models also show behavioral alterations relative to their non-transgenic littermates, so these animals allow the testing of potential therapies to improve AD behavior (Moran *et al*., 1995; Nalbantoglu *et al*., 1997; Chen *et al*., 2000; Boutajangout *et al*., 2010; Cramer *et al*., 2012). The behavioral tests typically measure hippocampal-dependent spatial-based learning and memory, as this type of behavior is altered in AD. Various environmental stimuli are tested, and the responses of the animals recorded. Although learning and memory cannot be directly observed, researchers can reasonably infer that learning has occurred if, for example, a mouse learns to swim towards a submerged platform marked with a flag.

A number of mouse behavior tests have been designed to test the effects of various drug therapies or environmental stresses on AD mice. Such tests include the Morris water-maze test, the Y-maze test, the radial arm maze test, the closed field symmetrical maze test, and the object recognition test. In this MQP project, the Morris water-maze test (Morris, 1984) was chosen to help evaluate a potential therapeutic drug, because the Morris test has potentially been shown to distinguish AD from WT mice (Moran *et al*., 1995). In the Morris test, a mouse is placed into a round pool of harmless opaque water containing a hidden platform, and trained to locate a visible escape platform. To demonstrate learning, the time spent locating the platform is recorded by a camera. Later, the flag marking the hidden platform is removed, and the experiment is repeated, recording the time spent locating the unmarked hidden platform. In other modifications of the swimming test, once a mouse has learned the position of an unmarked platform, the platform is moved, and the number of trials to learn the new position is recorded (Chen *et al*., 2000).

AD mouse research using the Morris water-maze test has yielded numerous research outcomes. Using a Morris water-maze test, transgenic AD mice have been reported to show a learning deficit between the ages of 6-12 months without signs of neurological failure or abnormal activity (Moran *et al*., 1995; Nalbantoglu *et al*., 1997). Another study modified the morris water-maze test to allow for long-term and short-term memory processing (Chen *et al*., 2000). In both cross-sectional and longitudinal studies, AD mice showed an age-related deficit in the amounts of trials required to learning the series of spatial locations (Chen *et al*., 2000). A study investigating the effects of tau immunotherapy on AD mouse behavior showed that the removal of pathological hyper-phosphorylated tau protein in AD mice improved their performance in a radial arm maze test, closed field symmetrical maze test, and a novel object

recognition test (Boutajangout *et al*., 2010). More recently, a Morris water-maze test showed improvement in hippocampal function of AD mice treated with bexartoene (Cramer *et al*., 2012).

Neurotrophic Factors

Neurotrophic factors (NTFs) are a family of diffusible proteins released by neurons and supporting cells, such as glia and oligodendrocytes, which play a pivotal role in the survival, development, maintenance, repair, and function of neuronal cells. During development, NTFs are involved in the formation of the normal synaptic network (Levy *et al.*, 2005) and promote axonal development and neuronal survival. Beyond development, NTFs also function to maintain specific neuronal functions, such as improving neuronal plasticity during aging and diseased states (Murer *et al.*, 2001; Levy *et al.*, 2005; Erickson *et al.*, 2010; O'Bryant *et al.*, 2011; Balaratnasingam & Janca, 2012).

Four main groups of NTFs have been identified in humans (**Figure-8**): including the Brain-Derived Neurotrophic Factor Family (BDNF), Nerve Growth Factor Family (NGF), Neurotrophin-3 Family (NT-3), and Neurotrophin-4 Family (NT-4).

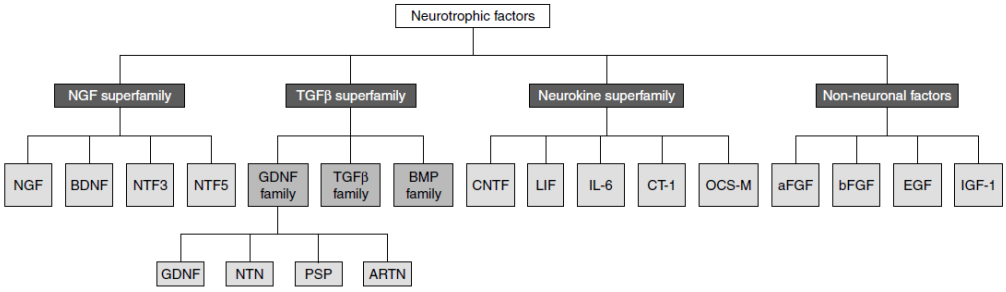


Figure-8: Diagram of the Four Main Human Neurotrophic Factor Families. **aFGF** = acidic fibroblast growth factor; **ARTN** = artenin; **BDNF** = brain-derived neurotrophic factors; **bFGF** = basic fibroblast growth factor; **BMP** = bone morphogenic protein; **CNTF** = cilliary neurotrophic factor-1; **CT-1** = cardiotrophin-1; **EGF** = epidermal growth factor; **GDNF** = glial cell line-derived neurotrophic factor; **IGF-1** = insulin-like growth factor; **IL-6** = interleukin-6; **LIF** = leukemia inhibitory factor; **NGF** = nerve growth factor; **NTF** = neurotrophin; **NTN** = neurturin; **OCS-M** = oncostatin-M; **PSP** = persephin; **TGF**β = transforming growth factor-β (Levy *et al.*, 2005)

Following the discovery of the first neurotrophic factor, nerve growth factor (NGF) in 1952 by Rita Levi-Montalcini (Levi-Montalcini, 1952; Levi-Montalcini, 1953), much research has gone into the roles of NTFs in neurodegenerative disease, and their potential use in therapeutic treatment. Research into these roles led to the formation of the *target-derived neurotrophic support concept*, which states that a lack of endogenous NTFs in specific neuronal populations causes the neuronal death observed in neurodegenerative dementias (Murer *et al.*, 2001; Levy *et al.*, 2005). Given that NTFs are often produced in areas affected by early-stage AD, NTFs are an attractive candidate for AD therapeutics, and many researchers are investigating their role as potential therapeutics for neurological disorders, including AD (Barinaga, 1994; Tuszyski & Gage, 1994; Hefti, 1997; Shen *et al.*, 1997; Zuccato & Cattaneo, 2009).

Early experimental studies involving the intra-cerebroventricular (ICV) infusion of neurotrophic factors suggested that NTF treatment partially reversed the physiological effects associated with AD; deficits in spatial memory were observed in the Morris water test in NGF 'knockout' mice and in mice containing antibodies to NGF (Levy *et al.*, 2005), while the intranasal administration of the NGF resulted in a complete restoration of neurons in the frontal cortex, reduced amyloid plaques, and reversed hyper-phosphorylated tau deposition (Capsoni *et al.*, 2002). Reversal of impaired memory or synaptic function has also been observed in studies where NTFs were delivered exogenously as pure proteins, or via gene therapy or stem cells (Blurton-Jones *et al.*, 2009; Nagahara *et al.*, 2009). Cell death due to exposure to toxins, brain trauma, or nerve injury were prevented by pharmacologically delivering NTFs (Jellinger *et al.*, 2002).

Ependymin (EPN) is a neurotrophic factor previously shown to be up-regulated in gold fish brain following a series of training and learning events (Benowitz & Shashoua, 1997). EPN has multiple functions including facilitating optic nerve elongation and long-term memory formation. Later discovered in mice, monkeys, and humans (Adams & Shashoua, 1994; Adams *et al.*, 1996; Apostolopoulos *et al.*, 2001), EPN became a possible AD therapeutic in our lab. However, instead of delivering full-length EPN, our lab uses short EPN mimetics (such as CMX-8933, CMX-9236, or hEPN-1) to facilitate crossing the blood brain barrier. Our lab has shown that goldfish EPN mimetics CMX-8933 and CMX-9236 can induce neuroprotection *in vitro* (Adams *et al.*, 2003) and *in vivo* (Shashoua *et al.*, 2003; 2004), and can upregulate anti-oxidative enzymes SOD-1, catalase, and glutathione peroxidase (Shashoua *et al.*, 2003; 2004). We have also shown that human hEPN-1 can restore human neuronal survival *in vitro* against an Aβ challenge (Stovall, 2006), lowers caspase activation (Kapoor, 2007), lowers tau hyper-phosphorylation *in vitro* (Ronayne, 2008), and lowers Cathepsin activity (Donahue & Lobdell, 2011).

Anti-Oxidative Enzyme: Superoxide Dismutase (SOD)

A prevalent theory regarding cell death is the free radical theory of aging, which states that reactive radicals result in severe cellular damage, and eventual cell death with continued exposure (Beckman & Ames, 1998; Venarucci *et al.*, 1999; Allen & Tresini, 2000). In Alzheimer's, Aβ causes a cellular increase in reactive oxidative species (ROS). Accordingly, the accumulation of ROS results in the brain's inability to produce anti-oxidative enzymes, such as catalase (CAT), glutathione peroxidase (GPX) or superoxide dismutase (SOD) (Smith *et al.*, 2000; Shahoua *et al.*, 2004).

SOD catalyzes the dismutation of superoxide anions to hydrogen peroxide. Hydrogen peroxide is then broken down to molecular oxygen and water by GPX or CAT (Sun and Chen, 1998). All three enzymes serve to protect the cell against free radicals and reactive oxygen. Studies have shown that over-expression of SOD make mouse models resistant to cerebral anoxia (Chan *et. al.*, 1998; Sheng *et al.*, 1999; Li *et al.*, 2001), as SOD serves a neuroprotective function. Previously, our lab showed that neuroblastoma cells show increased SOD levels following *in vitro* treatment with hEPN (Parikh, 2003). Our project will test whether the human NTF hEPN affects SOD levels in transgenic AD mouse models, and whether the potential SOD changes correlate with behavioral improvements.

PROJECT PURPOSE

Our laboratory previously showed that treatment of cultured human SHSY neuronal cells *in vitro* with a short neurotrophic factor mimetic increases neuronal cell survival and increases cellular levels of anti-oxidative superoxide dismutase (SOD-1). The purpose of this project was to determine whether intraperitoneal treatment of transgenic AD mice with this short peptide improves learning or memory, and if so, does the improved behavior correlate with an increase in the cellular levels of SOD. A Morris water swimming test, that requires cortical and hippocampal function, was used to potentially distinguish WT and AD mice treated with vehicle or peptide drug. Immunoblots were used to assay the levels of anti-oxidative SOD-1 in the brain to see if those levels correlated with behavior.

METHODS

In Vivo AD Mouse Model

Hemizygous male Alzheimer's mice were purchased from The Jackson Laboratory (strain #006554). This strain is 5X transgenic for human amyloid precursor protein (APP) and presenilin (PSEN) mutations. The Tg males were crossed with B6 WT females (strain #100012), so that approximately 50% of the offspring were AD and 50% WT. The pups were aged to 9 months, an age at which senile plaque formation is prevalent. Two WT sentinel mice were sent to Charles River Labs for histology testing to prove the colony was disease free. The aged mice were then dosed twice per day for two weeks i.p. with 60 mg/kg hEPN NTF mimetic, after which mice were sent to PsychoGenics (Tarrytown, NY) where the morris water-maze tests were conducted. Following the tests, the mice were euthanized, and the brains and tail sections were harvested and sent back to WPI and stored at -80ºC.

Morris Water-Maze Test

Prior to the test, mice were examined, weighed, and handled to minimize manipulation-associated stress, and to confirm test subjects were healthy and suitable for testing. A circular plastic maze (120 cm diameter) that contained external pool cues and 74-76^{o}C opaque water was used. The maze was divided into four quadrants marked by distant spatial cues. The water was made opaque through the addition of a non-toxic paint to hide an escape platform located 25 cm below the pool rim (0.5 cm below the water's surface). Mouse behavior was monitored using a video camera placed above the maze. Analysis was conducted using the Watermaze tracking package (Coulbourn, USA).

During day 1 and 2, mice were handled for 60 seconds to reduce stress associated with manipulation and testing. At the end of day 2, subjects were introduced to shallow water (74-76^{o}C) for 20 seconds. Behavioral testing began on day 3 and lasted for three consecutive days. A platform marked with a visible cue just above the surface of the water was placed roughly half-way between the center of the maze and the wall, in the center of one of the quadrants. Mice underwent six training trials, spaced at roughly 20 minute intervals.

During day 6 through 10, the visible cue at the platform was removed, and mice were required to locate the hidden unmarked platform during four trials, spaced at roughly 20 minute intervals. The platform location remained consistent throughout teach trial, requiring the animals to rely on the initial spatial cues to learn the platform location.

The time it took to locate each platform and the total distance traveled was recorded for each sample. Phase 1 of the trial (day 3-5) enabled a measurement of the animal's ability to locate and climb onto the flagged platform. Phase 2 of the trial (day 6-10) served to measure the animal's memory of that location. Fifty-six mice were tested in two cohorts. Tests were performed during animal light cycle phases, and without knowing whether the mouse was AD or WT.

Genotyping AD Mouse Tissues

The genotype of each mouse was determined by PCR for APP and PSEN transgenes in tail section DNA. DNA was isolated by phenol extraction techniques, then amplified with primers for human APP or PSEN (see below).

Phenol Extraction from Mouse Tails

DNA was extracted by first removing 0.5 mm of tail section from the tail segment provided to us by PsychoGenics, and placing it in a 1.5mL eppendorf tube. A 0.5 mL volume of freshly made DNA digestion buffer with proteinase K (50 mM Tris-HCl pH 8.0, 100 mM EDTA pH 8.0, 100 mM NaCl, 1% SDS, 0.5 mg/mL fresh proteinase-K powder) was added. Samples were incubated overnight with gentle shaking at 50-55^{o}C. Tubes were spun briefly to remove the solution from inside the cap, then a 0.7 mL volume of neutralized phenol/chloroform/isoamyl alcohol (25:24:1) was added. Tube was mixed for 5 minutes on clinical rotator. Following a 5 minute microfuge at top speed, 0.5 mL of the upper phase was transferred to a new microfuge tube. A 1 mL aliquot of 100% ethanol at room temperature was added. The tube was inverted until DNA precipitate developed. Tube was spun in a microfuge for 5 minutes at maximum speed, and the supernatant was carefully removed and discarded. 0.5-1.0 mL of 70% ethanol was added, and the sample was inverted numerous times to wash the DNA. Following a 5 minute microfuge at maximum speed, the supernatant was carefully removed and discarded. Tubes were spun again to remove any remaining ethanol solution. The sample was left overnight to dry at room temperature. The following day, DNA was resuspended by adding 200 µL of 1X TE buffer and incubating the sample for 15 minutes at 55^{o}C to dissolve the DNA. The samples were diluted 1:100 in TE buffer, for a final yield of 20-50 µg DNA.

PCR

Master mixes (400 µL) were prepared for ten APP and PSEN genotyping reactions (50 µL per reaction) according to the following table:

APP: • 237 µL of dH_2O • 50 µL of 10X PCR buffer • 25 µL of 50 mM $MgCl_2$ • 10 µL of 10 mM dNTPs • 25 µL of 20 µM APP-3610 • 25 µL of 20 µM APP-3611 • 12.5 µL 20 µM Ctrl-8744 • 12.5 µL of 20 µM Ctrl-8745 • 3 µL of 5 U / µL Taq Polymerase	**PSEN**: • 196 µL of dH_2O • 50 µL of 10X PCR buffer • 25 µL of 50 mM $MgCl_2$ • 10 µL of 10 mM dNTPs • 33 µL of 20 µM PSEN-1644 • 33 µL of 20 µM PSEN-1645 • 25 µL 20 µM Ctrl-7338 • 25 µL of 20 µM Ctrl-7339 • 3 µL of 5 U / µL Taq Polymerase

Master mix (40 µL) was aliquoted into the tubes, then 10 µL of tissue lysate was added to make a 50 µL reaction. The tubes were subjected to PCR as follows: Step 1: 94°C for three minutes; Step 2: 94°C for 30 seconds, 52°C for one minute, 72°C for one minute; Repeat Step 2 for 35 cycles; Step 3: 72 °C for two minutes; Step 4: 4 °C infinitely. Following PCR, 5.5 µL of 10X Sample Buffer (0.025% xylene cyanole, 100 mM EDTA pH 8.0, 50% glycerol) was added to each 50 µL reaction, then 10 µL was loaded onto a 2.5% agarose gel in 1X TAE buffer containing 1 µg/mL (final concentration) Ethidium Bromide. The first lane was loaded with 10 µL of 100 bp DNA ladder (0.5 µg). Gels were electrophoresed at approximately 60 V for 1.5 hr, then were photographed by UV trans-illumination.

Brain Whole Cell Lysates

A small slice of brain tissue (0.1 g) was combined with 1 mL of Complete Lysis Buffer [20 mM HEPES pH 7.9, 10 mM KCl, 300 mM NaCl, 1 mM $MgCl_2$, 0.1% Triton X-100, 20% glycerol, 0.5 mM DTT (freshly added; Gibco), and 0.5 mM PMSF (freshly added; Sigma)] in a 1mL dounce homogenizer. The sample was homogenized by at least ten up-and-down strokes. Homogenates were transferred to a 1.5 mL eppendorf tube and placed on ice. The sample was

microfuged for 5 minutes at 4^{o}C, at maximum speed to pellet cell debris. Aliquots of supernatant were stored at -80°C.

SOD & Tubulin Immunoblots

The Bradford Assay was used to determine the total cellular protein concentrations of each tissue lysate. Necessary volumes for 5 µg per lane for tubulin or 2 µg per lane for SOD were mixed with 1X protein sample buffer (0.125 M stacking gel buffer, 4% SDS, 20% glycerol, 10% β- Mercaptoethanol, and 0.1% bromophenyl blue powder) to make a total load volume of 10 µL. A 20 µL load volume of 1X sample buffer (10 µL)and Kaleidoscope Pre-Stained Standard (10 µL, Bio-Rad) was also made. Samples were boiled for two minutes to ensure protein denaturation and an even coating with the SDS. Samples were then loaded onto gels with a lower gel containing 10% polyacrylamide, 0.38 M resolving buffer, 0.1% SDS, 0.1% ammonium persulfate, and 0.05% TEMED. The upper gel was composed of 5% polyacrylamide, 0.125 M stacking gel buffer, 0.1% SDS, 0.1% ammonium presulfate, 0.1% TEMED. The protein electrode buffer used included 25 mM Trizma Base, 0.192 M glycine, and 0.1% SDS. Prior to sample loading, gels were pre-run for 15 minutes at 150 volts to equilibrate with the buffer, then electrophoresed for roughly 150 volts for three hours.

Following electrophoresis, the protein was electroblotted to nitrocellulose membrane (0.45 µm pore size, Whatman) in pre-chilled transfer buffer (48 mM Trizma Base, 39 mM glycine, 0.037% SDS, 20% methanol). Transblotting occurred at 50 volts for two hours at 4°C with stirring. Membranes were then submerged in PBS-CT western blocking solution (1% casein, 1X PBS, 0.2% Tween-20) with the membrane protein-side up. The membrane was blocked at room temperature for at least one hour with rocking.

Primary antibody incubations for SOD contained 25 μL of 90 mg/ml rabbit anti-bovine SOD-1 (Rockland) added to 50 mL of fresh blocker solution (1:2000 dilution), to give a final concentration of 45 μg/mL. Primary antibody incubations for β-tubulin contained 25 μL of 500 μg/ml anti-β-tubulin (Imgenex, IMG-5810A) mixed with 50 mL of fresh blocker solution (1:2000 dilution) to give a final concentration of 0.25 μg/mL. Membranes were incubated with primary antibody for two hours at room temperature with rocking, then washed twice for two minutes with PBS-Tween (1X PBS, 0.05% Tween) using vigorous shaking on a gyratory shaker. Secondary antibody incubations included a 0.4 mg/mL final concentration of goat-anti-rabbit-HRP (Pierce) and a 0.5 mg/mL final concentration Streptavidin-HRP (Pierce; 1:1000 dilutions of glycerol stocks; 25 μL of secondary antibody added to 25 mL blocker). Secondary antibody incubations were performed for two hours at room temperature with gentle shaking. Membranes were then washed three times with PBS-Tween for two minutes with vigorous shaking on a gyratory shaker, then rinsed briefly with 1X PBS before detection.

SuperSignal West Pico chemiluminescent substrate (Pierce) was used to detect protein presence by combining equal amounts of Stable Peroxidase Solution and Luminol/Enhancer Solution just prior to use, and incubating the membrane at room temperature in the fresh substrate solution for five minutes with the protein-side facing upward. The substrate was then allowed to drip from the membrane without drying, then placed between two clear plastic sheets (Gibco) in a film cassette. Tubulin blots were exposed to Biomax XAR-5 film (Kodak) for four minutes, and SOD blots for four seconds. Film was developed automatically in the Kodak M35A X-Omat Processor.

Human SH-SY5Y Cell Culture

Human SH-SY5Y neuroblastoma cells (commonly termed SHSY) were obtained from medium-adapted liquid nitrogen stocks previously obtained from ATCC and prepared in our laboratory. Culture medium contained 500 mL DMEM-F-12 (ATCC), 50 mL of FBS (Gibco, ATCC, or Hyclone) to give a final concentration of 10%, and 0.275 mL of 10 mg/mL gentamycin (BioWhittaker) to give a final concentration of 5 μg/mL. The DMEM-F-12, FBS, and gentamycin were mixed in the 500 mL medium bottle, then filter sterilized. Medium was stored at 4°C.

To thaw our lab's SHSY cells from liquid nitrogen storage, the cells were placed in a 37°C water bath for one to two minutes until completely thawed, then carefully re-suspended and transferred into a T-25 flask where 5 mL of pre-warmed medium was added. The plate was placed in a 37°C + 5% CO_2 humidified incubator. Following 24 hours, old medium was replaced with fresh pre-warmed media.

Cultures were fed every 3-4 days until the flasks achieved approximately 80% confluency, at which point cultures were split 1:2 into new flasks. To feed cultures, the old medium was aspirated and replaced with 4 mL of fresh, pre-warmed medium. To split cultures, the old medium was aspirated and replaced with pre-warmed medium (8 mL for T-25), and the flask scraped to release the cells from the floor of the flask. The cells were then re-suspended and pipetted into new flasks (4 mL for T-25).

To freeze SHSY cells, confluent T-75 flasks were scraped and the cell suspension centrifuged for five minutes at 6500 rpm. The supernatant was aspirated, and 1 mL of pre-warmed freezing medium (Gibco) was added. The cells were re-suspended, pipetted into a

freezing vial, and stored at -80°C in a Styrofoam® rack for insulation to slow the freezing process. After 24 hours, the cells were moved to liquid nitrogen storage.

For plating experiments, 80% confluent T-25s (2 plates) were split and plated onto four T-25s. For Aβ treatment conditions, Yankner peptide (Tocris; see below) was introduced to flasks at 20 µM (80 µL of 1 mM stock per 4 ml medium). For hEPN treatment conditions, hEPN-1 peptide (BioTherapeutix, Inc.) was introduced to flasks at 150 µM (160 µL of 3.75 mM stock per 4 ml medium). Untreated, control flasks generally reached approximately 80% confluency after 72 hours, at which point cells were subjected to morphology and trypan blue exclusion cell counts (see below) and harvested by scraping.

Yankner Peptide

Human Yankner peptide powder ($A\beta_{25-35}$) was purchased from Tocris Bioscience (#1429, Batch 5B). 1 mM stock solutions were prepared by dissolving 1 mg of powder (943 nmol) per 0.94 mL of 1 mM sodium bicarbonate. Stock solutions were stored at -20°C. The Yankner peptide was used at a final concentration of 20 µM (80 µL of the 1 mM stock per 4 ml of medium) in human SHSY cell culture, which was previously shown in our lab to produce neurotoxic effects.

hEPN-1 Neurotrophic Factor

Several human ependymin–1 (hEPN-1) peptides containing the same amino acid sequence in different salt conditions were provided by BioTherapeutix, Inc. (Brookline, MA) or CS Bio Company (Menlo Park, CA). Peptides were received as dry powders and stored at

-20°C. For plating experiments, hEPN-1 peptides were prepared as 3.75 mM stock solutions by dissolving 1 mg of peptide per 1 mL of filtered DMEM-F-12 medium without serum. 160 µL of the 3.75 mM stock was added to 4 ml medium in a T25 flask to make a 150 µM final concentration.

Cell Morphology Counts

Three representative regions of each experimental flask were imaged at 72 hours post-plating by a Leica inverted microscope and camera attachment at 20x magnification. Images were used to count cells with free stellate morphologies, physically networked morphologies, and free non-stellate (lacking protrusions, or "balled up") morphologies, as well as total cells per viewing field. Counts from all three representative regions of flasks were averaged to provide final morphology estimates as a percentage of total cell counts.

Trypan Blue Exclusion Viability Assay

Cell suspensions harvested from experimental flasks were vortexed, and 10 µL aliquots of suspension were transferred to a 1.5 mL eppendorf tube. Each aliquot was vortexed and pipetted onto a clean microscope slide with an equal volume of 0.4% Trypan Blue (Gibco), and the solution was mixed using a pipette tip and spread to create an area of about 1cm by 1cm. Three representative regions of each slide were imaged (as described above), and images were used to count total viable (Trypan Blue-excluding) and non-viable cells. Counts from all three representative images were again averaged to provide final viability estimates as a percentage of total cell counts.

RESULTS

The purpose of this project was to identify a behavioral test that was effective in distinguishing AD from WT mice, to determine whether hEPN-1 treated mice perform better in the test, and to determine whether the behavior correlates with changes in brain SOD levels.

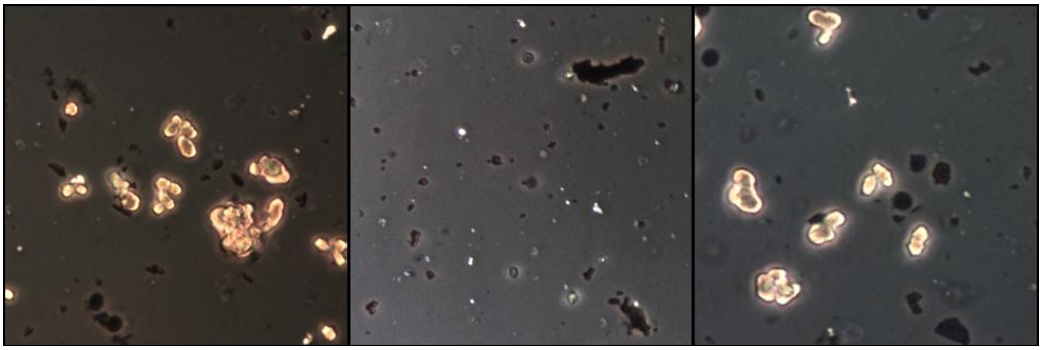
In Vitro Alzheimer's Model with Aβ and hEPN

Our lab previously showed that adding Aβ to human SHSY cells in culture is an effective *in vitro* model of AD, as the addition increases cell death (Stovall, 2006), tau hyper-phosphorylation (Ronayne 2008), apoptotic TUNEL staining (Ronayne 2008), and caspase-3 levels (Kapoor, 2007). When simultaneously adding hEPN with the Aβ, these effects all decreased. This in vitro system was used to validate the drug used in vivo.

Trypan Blue Exclusion Test of Cell Viability

To test whether the hEPN therapeutic could rescue function in neuronal cells, an *in vitro* assay was set up using an SHSY neuronal cell line. At time of splitting, one flask of cell culture received 20 μM Aβ, another received Aβ + hEPN1 BTX-639, and a third flask was left untreated as a control. When the control flask reached 80% confluency, the cells were treated with trypan blue staining to monitor cell viability. Dead cells cannot exclude the dye so appear dark blue. Viable cells exclude the dye and appear white. hEPN was effectively shown to reverse the effects of Aβ (**Figure 9**)**.** Compared to the control flask, the Aβ-treated flask showed minimal neuronal growth, and the addition of the hEPN resulted in restoration of the cells. A cell count analysis (**Figure-10**) showed that the simultaneous addition of Aβ + hEPN was able to increase

cell viability by 30%, bringing the total cell viability to near normal conditions (normal=73%, hEPN+Aβ = 68%). Thus, this data illustrates that the hEPN therapeutic is effective *in vitro*.



Untreated **Aβ** **Aβ + hEPN**

Figure-9: Trypan Blue Analysis of Human SHSY Cell Viability. The three photomicrographs show an untreated (control), Aβ treated (20 µM), or Aβ (20 µM) and BTX-639 (150 µM) treated flask. Cells were harvested, stained with Trypan blue, and viewed under 20X magnification.

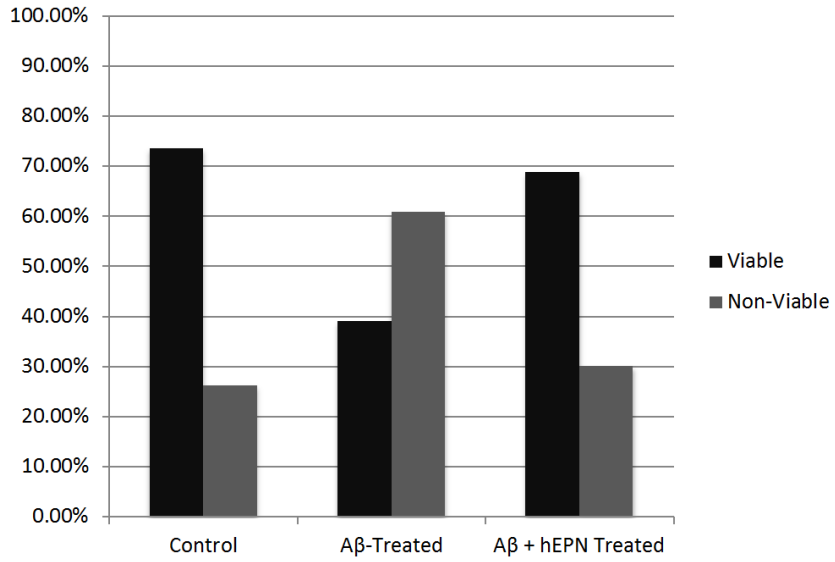


Figure-10: Cell Viability Analysis. The bar graphs represent the percent viable/non-viable cell counts from the *in vitro* cell culture experiment. Black: viable, gray: non-viable.

Mouse Genotyping

Because hemizygous AD males were bred to WT females, about 50% of the offspring are AD and 50% WT. The genotype differences cannot be visibly distinguished, so genotyping was performed on tail section DNA by PCR for the presence of the human APP and PSEN transgenes. Genotyping was performed on 52 mice; APP and PSEN transgenes were measured separately, then the results were compared (**Figure 11**).

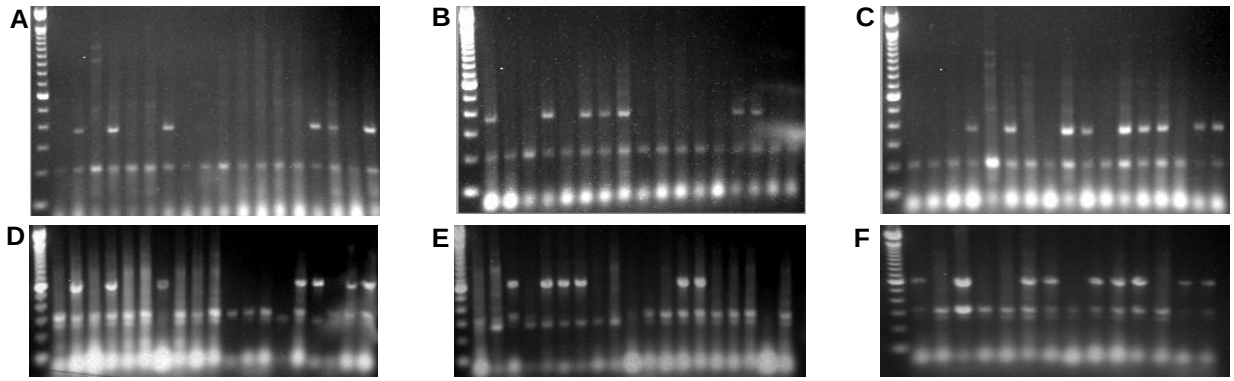


Figure-11: Mouse Genotyping. Tail section DNA for 52 mice was isolated and amplified by PCR for human APP or PSEN transgenes. Amplified DNA was electrophoresed on 2.5% agarose gels and photographed with UV trans-illumination. **A-C)** APP genotyping for 52 mice. **D-F)** PSEN genotyping for 52 mice. Main amplicon (APP or PSEN) is the strong band in the middle. The lower band represents the internal control for each reaction. Mice that showed positive for APP mutations were also positive for PSEN mutations.

Figure-12 shows a summary table of the 36 mice chosen by PsychoGenics for the morris swimming test. They divided their mice into two cohorts. Rougly 20% of those tested in the first cohort were AD mice, while in the second cohort an estimated 56.25% of mice were AD.

A

Subject	APP (+)	PSEN (+)	Classification
1	-	-	WT
2	-	-	WT
3	-	-	WT
4	-	-	WT
5	-	-	WT
6	-	-	WT
7	-	-	WT
8	+	+	AD
9	-	-	WT
10	+	+	AD
11	-	-	WT
12	-	-	WT
13	-	-	WT
14	-	-	WT
15	+	+	AD
16	-	-	WT
17	+	+	AD
18	-	-	WT
19	-	-	WT
20	-	-	WT

B

Subject	APP (+)	PSEN (+)	Classification
21	+	+	AD
22	+	+	AD
23	+	+	AD
24	-	-	WT
25	+	+	AD
26	-	-	WT
27	-	-	WT
28	-	-	WT
29	+	+	AD
30	+	+	AD
31	-	-	WT
32	+	+	AD
33	+	+	AD
34	+	+	AD
35	-	-	WT
36	-	-	WT

Figure-12: Summary Table of Mouse Genotyping For the 36 Mice Tested by PsychoGenics. PsychoGenics performance test data included 36 of the original 52 genotyped mice. For performance data analysis, mice 1-4, 8, 10, 15 17, 21-32, 35, and 36 were used. Mice were divide into two cohorts, the first of which received a placebo (vehicle) while the second received the hEPN therapy.80% of mice in the first cohort were WT. In the second cohort, 43.75% of mouse subjects were WT. **A)** Cohort 1, Mouse subjects 1-20. B) Cohort 2, Mouse subjects 21-36.

Morris Water Maze Test of Mouse Cognitive Performance

Due to the uneven distribution of AD and WT mice within each cohort, the number of mice for data analysis was reduced. The first four WT mice in the first cohort were used for performance analyses, to match the number of available AD mice. In the second cohort WT mice represented a smaller percentage of the group, containing only 7 mice. Thus for data analysis, only the first seven AD mice were used.

As described above, mouse subjects were divided into two cohorts and observed using a camera placed above the plastic maze. Mice were trained to locate a hidden platform placed within one of the quadrants, which was located 0.5cm below the surface of the water. In the first three days of testing, the platform was marked with a visible flag cue. The visible cue was

removed during the next five days of testing. The time it took for each subject to remember to locate the hidden platform and the distance traveled was recorded.

When subjects were required to locate a marked platform, vehicle-treated AD mice located the platform between 32-46 seconds on day 1, and 22-30 seconds on the day 3 (**Figure-13, A**) (averaging 40 seconds on day-1 and 25 seconds on day-3, as each mouse typically did better each day) (**Figure-13, B**). In the same test, WT mice located the platform within 7-14 seconds on day-1 and 5-8 seconds on day-3 (**Figure-13, A**) (averaging 10 seconds on day-1 and roughly 6 seconds on day-3 (**Figure-13, B**). Similar results were obtained for unmarked platform tests. AD mice took on average 25 seconds to locate the hidden platform on the final day of testing, while WT located the platform in roughly 10 seconds ($p = 0.05$, **Figure-13, D**). Considering the significance of the data obtained ($p < 0.05$) as well as the clear time gap between AD and WT groups, the morris water maze test was identified as an accurate test for distinguishing AD and WT mice.

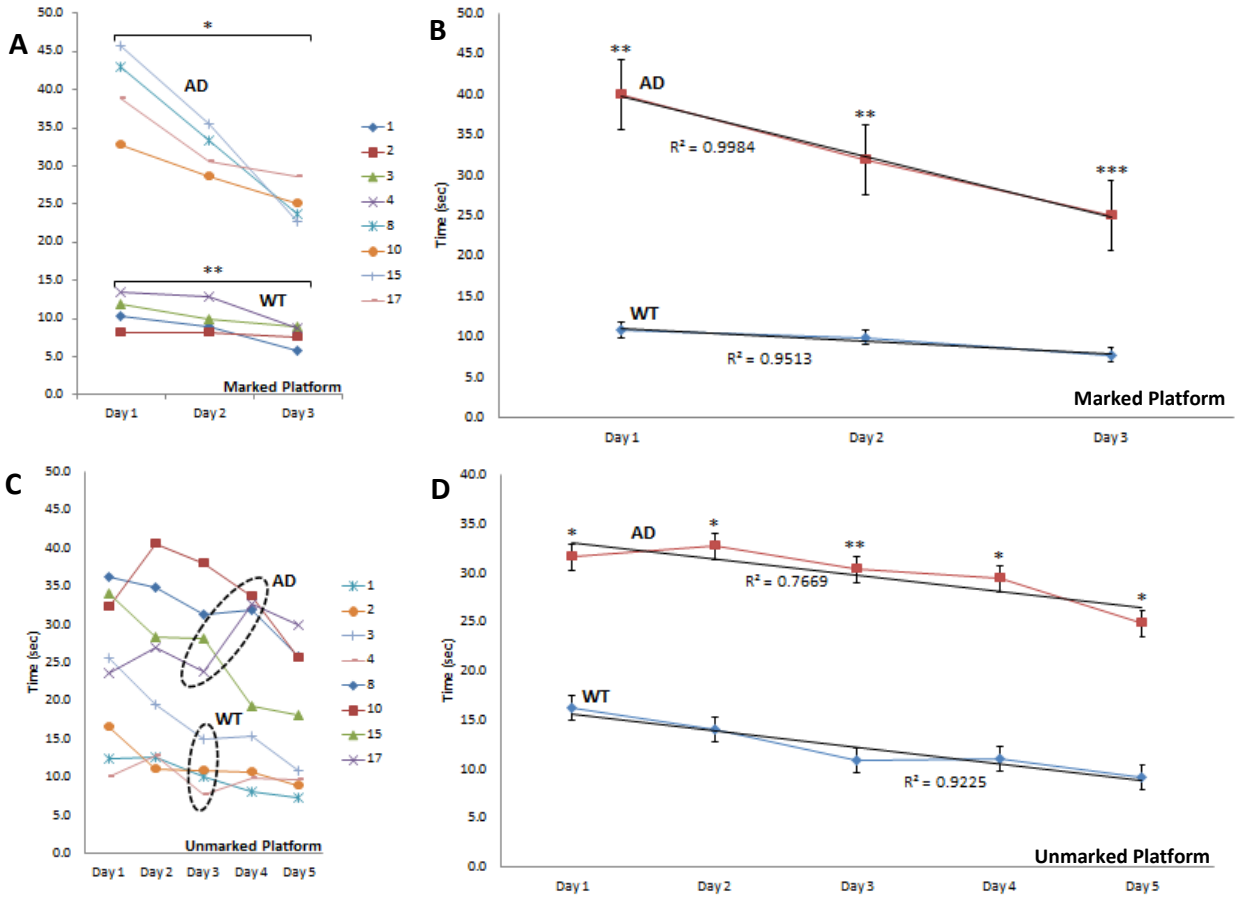


Figure-13: The Morris Water Maze Test Effective Distinguishes WT from AD Mice. After being acclimatized to handling and water, mice were tested over a three day period using a marked platform and 5-day period using an unmarked platform. Researchers conducted the test blind, thus they did not know which mice were AD or WT. Following genotyping, behavioral test results were matched with genotyping results. Platforms were marked with a flag, visible above the water. **A)** Time for each vehicle-treated mouse subject to locate marked platform over three days. The flag was removed in unmarked situations. **B**) Average time for AD and WT mice to located marked platform. **C)** Time for individual vehicle-treated mouse subjects to located unmarked platform. **D)** Average time for vehicle-treated mice to locate unmarked platform over 5 days.

After establishing that the morris water maze test provided an accurate distinction between AD and WT mice, analyses were done to determine the effectiveness of the hEPN treatment on AD mice. For this, vehicle and hEPN-treated AD mice were compared (**Figure-14**). Data showed that on average vehicle-treated AD mice took longer to locate marked and unmarked platforms than hEPN-treated AD mice ($p = 0.01$). hEPN treated mice took roughly 12

seconds to locate marked and unmarked platforms on final testing days (**Figure-14, C, F)**. Vehicle-treated mice, needed approximately 25 seconds to located marked and unmarked platforms on day 3 or day 5 of each test (**Figure-14, C, F**). Noting that the time for hEPN treated AD mice to locate a hidden platform approached the time required by WT mice, the therapeutic effectively improves AD mouse cognitive performance at the 60 mg/kg dose administered.

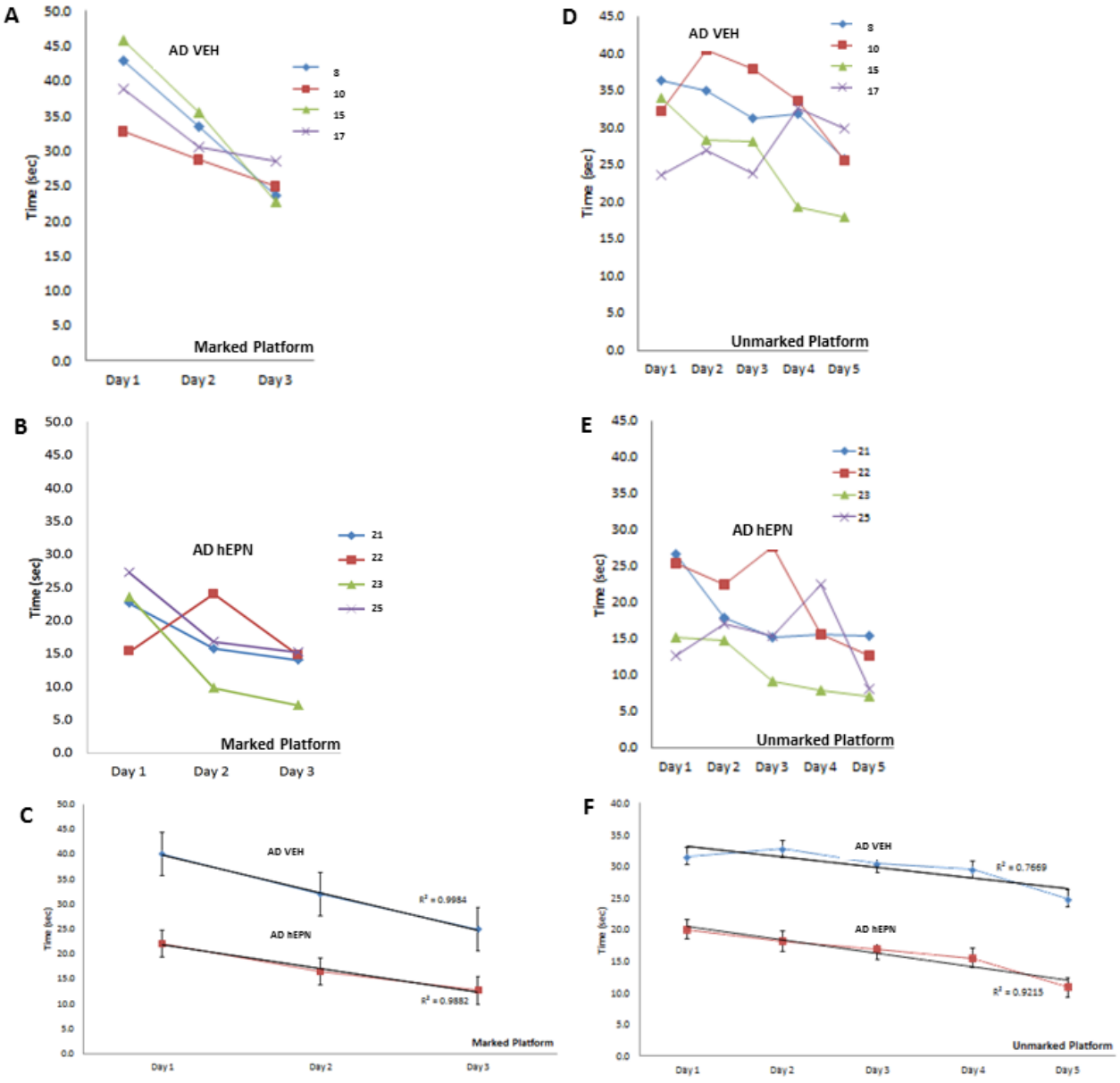


Figure-14: hEPN is an Effective AD Treatment. Morris water maze test results were compared between vehicle-treated AD mice and hEPN-treated mice. The time to located marked and unmarked platforms were recorded and compared. The left column displays results for vehicle and hEPN-treated mice locating a marked platform. The right column displays results for vehicle and hEPN-treated mice locating an unmarked platform. **A)** Mouse subjects 8, 10, 15 and 17 received a vehicle treatment, and took 32-46 seconds to locate the marked platform on day 1. Mice located the marked platform in roughly 25 seconds on day 3. **B**) Time for hEPN-treated mice (21, 22, 23 ,25) to located marked platform. **C)** Average time for vehicle-treated and hEPN-treated mice to located marked platform over three days **D)** Time to locate unmarked platform for vehicle-treated AD mice. **E)** Time to located unmarked platform for AD hEPN-treated mice. **F)** Average time for vehicle and hEPN treated mice to located unmarked platform.

Considering AD mouse performance was improved by the drug, it was hypothesized that the drug may also improve WT mouse performance. In a comparison of the effects of hEPN treatment on WT mice (**Figure-15**), it was found that hEPN1 treatment enhances WT cognitive performance. In marked and unmarked platform situations, hEPN-treated mice located the platform 1-3 seconds faster than vehicle treated mice on the final testing day (**Figure-15, A, B**). Overall, by the final testing day treated mice also traveled approximately 110 cm less to locate the marked platform (**Figure-15, C**), and roughly the same distance to the unmarked platform (**Figure-15, D**).

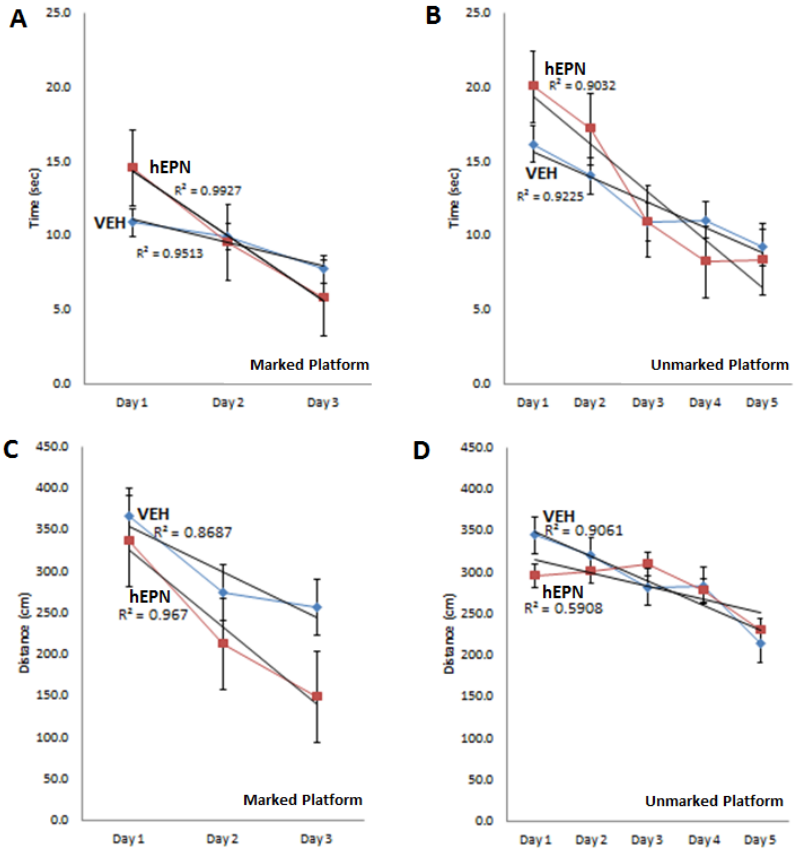


Figure-15: hEPN Slightly Enhances WT Mouse Performance. WT mouse performance was improved by hEPN treatment. Analysis was done comparing hEPN-treated and vehicle-treated WT mice. Altogether, WT mouse performance was improved by treatment with hEPN. **A)** Average time for mouse subjects to located marked platform. **B)** Average time for mouse subjects to located unmarked platform. **C)** Average distance traveled by mice to marked platform. **D)** Average distance traveled by mice to unmarked platform.

All mice, WT or AD, typically improve their swimming with each training. When reviewing the percent improvement in time or distance traveled over three (for marked platform) or five days (for unmarked platform), hEPN was further shown to improve both the time it takes for a mouse subject to located a platform and the distance traveled by each subject to a particular platform. Among WT or AD mice, cognitive performance increased between vehicle-treated subjects and hEPN treated subjects (**Figure-16**). Among vehicle-treated subjects, in general AD mice showed less performance improvement than WT mice (**Figure-16, A, C**). Among the hEPN treated mice, the greatest performance improvement was observed in WT mice (**Figure-16, B, D**). All data were analyzed using a standard t-test and standard error.

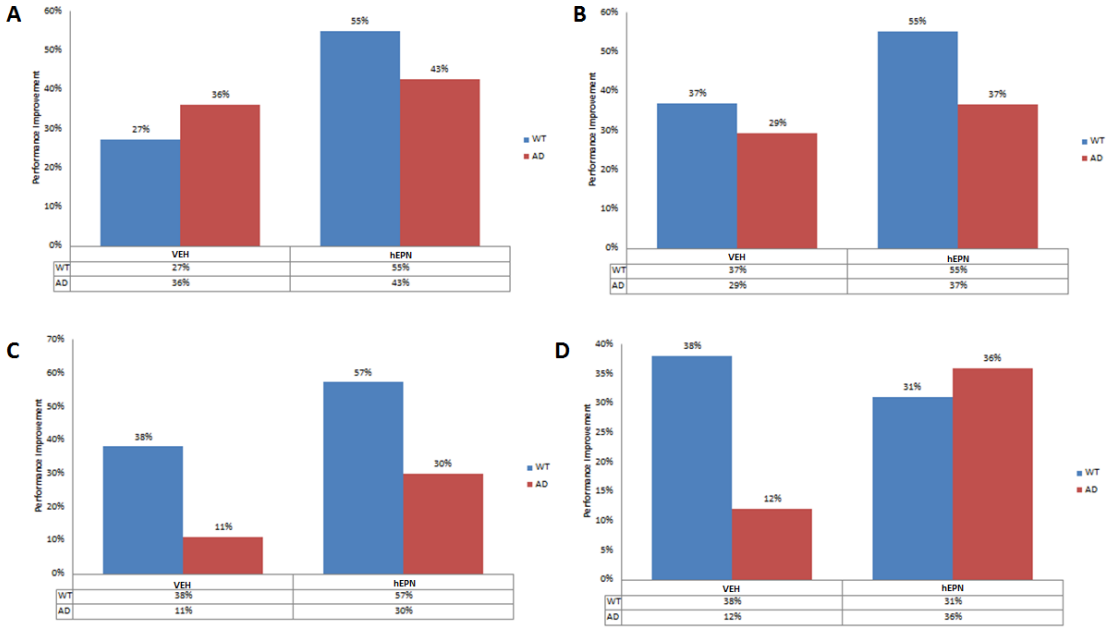


Figure-16: hEPN Enhances WT & AD Mouse Performance. The time it took for WT and AD mice to locate a platform on day 1 and day 5 was compared to determine the overall performance improvement for each subject. The percent improvement for each subject was averaged among WT and AD groups. It was found that in all situations, mouse cognitive performance improved with treatment. **A)** Percent improvement in time to locate marked platform.AD mice showed less performance improvement than WT-hEPN treated mice. AD vehicle-treated mice showed greater performance improvement than WT mice. **B)** Percent improvement in time to located to unmarked platform. AD mice showed less improvement than WT mice among vehicle and hEPN treated mice. **C)** Percent improvement in distance traveled to marked platform. AD mice showed less improvement than WT mice given vehicle and hEPN. **D)** Percent improvement in distance traveled to unmarked platform. AD vehicle treated mice showed less improvement than WT mice, however hEPN-treated AD mice showed more improvement than WT hEPN-treated mice.

SOD Immunoblots

Determining that hEPN treatment significantly improved AD mouse performance, preliminary investigations into the cause of the improved behavior were conducted. Because ROS is well known to increase in AD, it was hypothesized that cellular levels of SOD, an enzyme known to reduce ROS, would be greater in WT mice that show lower ROS. In line with previous research done in our lab showing that SOD levels increased with treatment of hEPN *in vitro*, a similar increase in SOD levels was expected in the mouse model.

To determine whether the *in vivo* i.p. hEPN therapy influenced the brain levels of SOD, immunoblots using extracts from AD vehicle (n=4) and AD therapy (n=4) mice brains were performed. Once brain tissue was lysed, it was separated by PAGE and blotted onto a nitrocellulose membrane. It was then probed using antibodies for both SOD (55 kDa) and tubulin (16 kDa). Scion Image software was used to analyze the mean optical densities (data not shown), which indicated that AD therapy mice showed a slight decrease in SOD levels in one set of results, although insignificant (**Figure-17**). This result was different from our stated hypothesis that SOD levels will increase with hEPN treatment. In the process of standardizing SOD and tubulin levels, it was observed that when SOD was standardized relative to tubulin, the tubulin levels actually strongly fluctuated between the samples. This might be the effect of hyperphosphorylated tau protein destabilizing the tubulin structures in the Aβ cascade, thus tubulin might not be the best marker to normalize the data against. Although hEPN might lower SOD levels *in vivo* slightly, there does not seem to be a major effect.

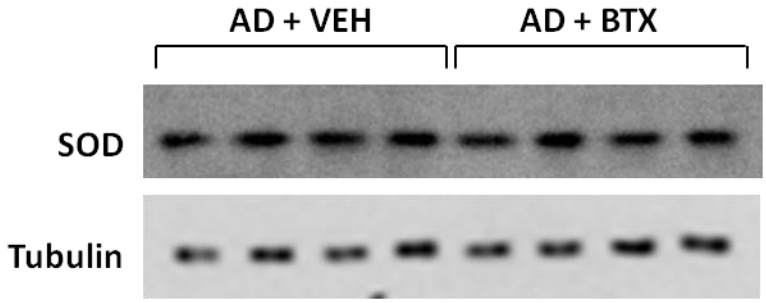


Figure-17: SOD Immunoblots for SOD and Tubulin. A. Immunoblot results of AD vehicle (n=4) and AD therapy (n=4) mice. Protein was detected by SuperSignal West Pico chemiluminescent substrate (Pierce), and exposed to Biomax XAR-5 film (Kodak) for four minutes for tubulin or one second for SOD. Film was developed automatically in the Kodak M35A X-Omat Processor.

DISCUSSION

This project extended our lab's previous *in vitro* and *in vivo* AD experiments by investigating the effects of neurotrophic factor treatment on AD mouse behavior. Previous research showed that treating human neuroblastoma cells *in vitro* with goldfish EPN partially restored cell viability (Stovall, 2006) and decreased tau hyper-phosphorylation and TUNEL staining (Ronayne, 2008) following an Aβ threat. Furthermore, our lab previously showed that cellular levels of antioxidant superoxide dismutase-1 (SOD-1) increased *in vitro* with hEPN1 treatment. This project extended these studies by examining the effects of hEPN in a 5X transgenic mouse model. A particular focus was placed on SOD as a potential cause of improved cognitive performance.

In Vitro Alzheimer's Model

An *in vitro* neuronal cell viability assay using trypan blue staining has shown that hEPN restores function in cells treated with toxic Aβ. The assay performed in this project demonstrates that the solubilized Aβ is indeed toxic to human neuronal cells, and that the cells can be rescued by the coapplication of hEPN + Aβ. It would have been also interesting to extend these experiments to add the hEPN at specific time points after the cells have been treated with Aβ to determine how late the NTF can rescue the programmed death pathway. This is important to consider since studies have shown that Aβ begins to accumulate in the brain of an AD patient years before the presence of symptoms.

Morris Water Maze Test

To date, researchers have tried a variety of behavioral assays to characterize and differentiate WT and AD mouse behavior, including the Y-maze, Rotarod, Elevated Plus-Maze test, and others. However, many of the tests show minimal differences between AD and WT mouse activity, suggesting the tests cannot easily distinguish the two groups. One particular study, that involved the use of transgenic female mice homozygous for human *β-APP*, showed the Morris test to be effective in distinguishing AD and WT mice (Moran *et al*. 1995). Our research shows that the Morris test can accurately distinguish 5X transgenic AD and WT mice at 8 months of age ($p < 0.05$). Furthermore, it shows that AD and WT mouse performance improves with treatment of hEPN-1 ($p = 0.01$).

SOD

It is known that SOD is typically up-regulated during cellular stress. We were interested in determining whether hEPN affects the levels of SOD in AD mice, and hypothesized that hEPN will increase SOD levels. Experimenting with 5X transgenic, 8-month old AD mice, we did not observe any significant changes in SOD levels when comparing AD vehicle to AD hEPN mice. This result may indicate that hEPN does not affect the cellular stress pathway, or that the hEPN1 dose and time used did not affect SOD levels, but more repeats of this experiment should be performed to make this conclusion. Previous studies in the lab have associated hEPN with increases in the levels of catalase and glutathione peroxidase, enzymes that function similar to SOD.

Future Recommendations

Currently, the cause of improved cognitive performance in mice as a result of hEPN-1 treatment is unknown. Future studies should involve a repeat of the previous behavioral tests using an appropriate pre-scoring mechanism to assess overall swimming capability and potentially measure AD and WT nest building ability. The study should include all bred mice in the data, to increase statistical significance. Upon repeat of the test and data analysis, a series of *in vitro* assays should be undertaken to understand the mechanistic nature of Aβ-induced cell death. Although SOD did not appear to be a major player in observed improvements in spatial learning and awareness, a variety of other brain proteins could be involved, many of which have been shown to be upregulated with hEPN treatment previously in our lab, such as anti-oxidant enzymes glutathione peroxidase (GPX) and catalases (CAT), known to serve a neuroprotective function in models for cerebral ischemia (Smith *et al.*, 2000; Shashoua *et al.*, 2001, 2003, 2004; Adams *et al.*, 2003), as well as cellular inhibitors of apoptosis (cIAPs), known to inhibit caspases and pro-apoptotic proteins. Our lab previously showed cIAPs are elevated post-treatment with hEPN (Rawal, 2010). Our lab's working hypothesis regarding the mechanism by which Aβ affects neuronal cells can provide a variety of other potential targets for investigation (**Figure-18**).

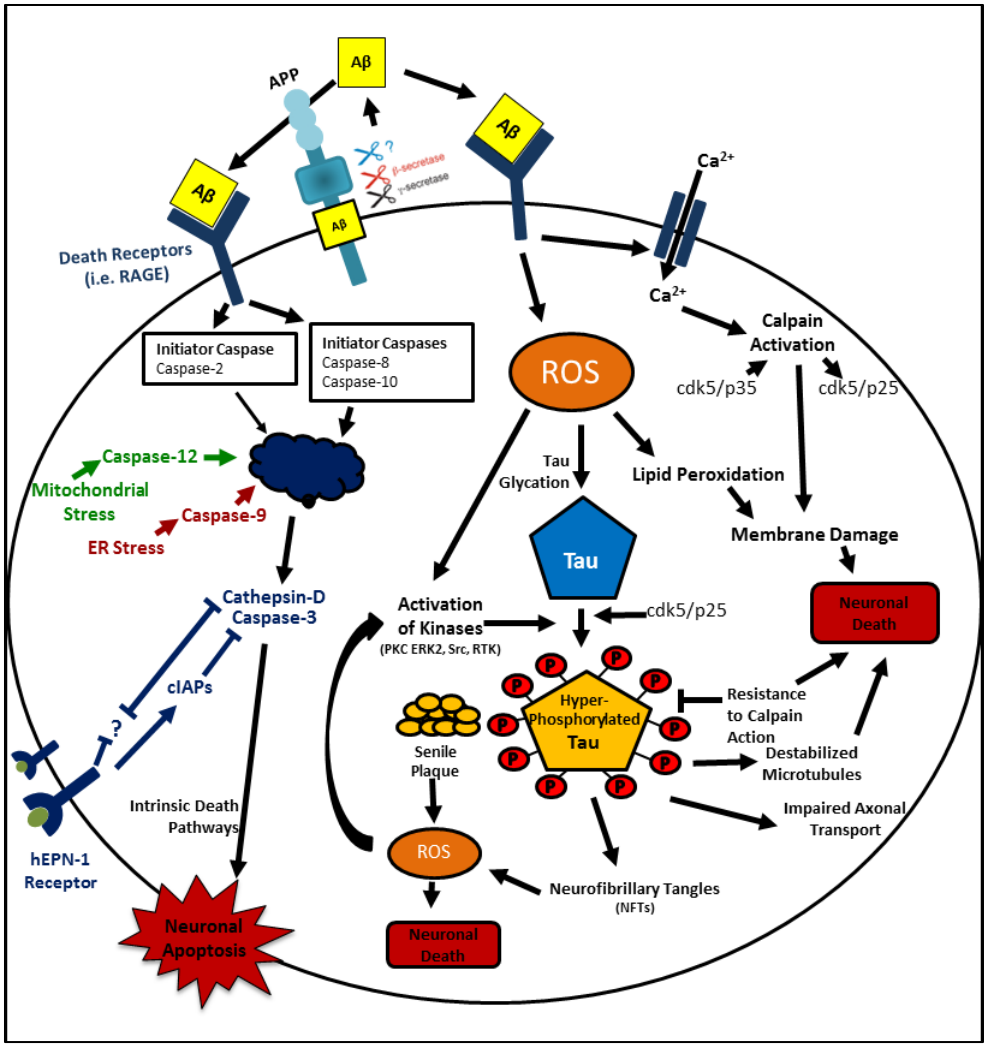


Figure-18: Summary of Our Lab's Previous Work. The diagram presents a combination of known aspects of the amyloid cascade hypothesis and findings made in our lab involving treatment with hEPN. Inaccurate cleavage of the amyloid precursor protein receptor (APP) by γ- and β-secretases initiates the Aβ cascade, through the production of a 40-42 amino acid toxic protein (Aβ). Our lab found that the Aβ peptide both increased caspase-2 and caspase-3 (Kapoor, 2007), and tau hyper-phosphorylation, as well as TUNEL staining (Ronayne, 2008). Overall Aβ was shown to increase cell death, while hEPN treatment partially restored theses effects. A portion of the hEPN mechanism involves the up-regulation of cellular inhibitors of apoptosis (cIAPs) (Rawal, 2010), potentially through the use of a separate receptor. Cathepsin-D, a lysosomal aspartic protease, is also involved in the cell death pathway, though its role is still unclear (Donahue and Lobdell, 2011). (*Derived from Adams Review; Donahue & Lobdell, 2011*).

The observed changes in tubulin levels in AD mice, when SOD levels appeared to remain constant between mice, is likely due to the effects of Aβ on tubulin polymerization. For this

reason, tubulin is likely an inaccurate housekeeper for AD mice. Investigations into the degree of polymerized tubulin in WT, Aβ-treated, and Aβ+hEPN-treated cells should be conducted to better understand the physiological effects of Aβ on tubulin and cellular shape. A more accurate housekeeper molecule should also be identified and used in future investigations.

BIBLIOGRAPHY

Adams D, Shashoua V (1994) Cloning and sequencing the genes encoding goldfish and carp ependymin. *Gene*, 141: 237-241.

Adams D, Kiyokawa M, Getman M, Shashoua V (1996) Genes encoding giant danio and golden shiner ependymin. *Neurochemical Research*, 21: 377-384.

Adams DS, Hasson B, Boyer-Boiteau A, El-Khishin A, Shashoua VE (2003) A peptide fragment of ependymin neurotrophic factor uses protein kinase-C and the mitogen-activated protein kinase pathway to activate c-Jun N-terminal kinase and a functional AP-1 containing c-Jun and c-Fos proteins in mouse NB2a cells. *Journal of Neuroscience Research*, 72: 405-416.

Allen RG, Tresini M (2000) Oxidative stress and gene regulation. *Free Radical Biology and Medicine*, 28: 463-499.

Alzheimer's Association (2012) http://www.alz.org/

Alzheimer's Disease Facts and Figures (2011) Alzheimer's & Dementia, Volume, Issue 2. http://www.alz.org/downloads/Facts_Figures_2011.pdf

Alzheimer's Disease Facts and Figures (2010) Alzheimer's & Dementia, Volume 6. http://www.alz.org/documents_custom/report_alzfactsfigures2010.pdf

Alzheimer's Disease International (2010) World Alzheimer's Report 2010: the global economic impact of dementia. http://www.alz.co.uk/research/files/WorldAlzheimerReport2010.pdf

Alzheimer's Foundation of America (AFA) (2010) About Alzheimer's. Retrieved 15 February, 2011, from http://www.alzfdn.org/AboutAlzheimers/definition.html

Apostolopoulos J, Sparrow RL, McLeod JL, Collier FM, Darcy PK, Slater HR, Ngu C, Gregorio-King CC, Kirkland MA (2001) Identification and characterization of a novel family of mammalian ependymin-related proteins (MERPs) in hematopoietic, nonhematopoietic, and malignant tissues. *DNA Cell Biology*, 20: 625-635.

Avila J, Lucas J, Pérez M, Hernández F (2004) Role of Tau Protein in Both Physiological and Pathological Conditions. *Physiol Rev*, 84: 361–384. http://physrev.physiology.org/content/84/2/361.short

Bachman DL, Wolf PA, Linn RT, Knoefel JE, Cobb JL, Belanger AJ, White LR, D'Agostino RB (1993) Incidence of dementia and probable Alzheimer's disease in a general population: The Framingham Study. *Neurology*, 43(3 Pt 1): 515–519. http://www.ncbi.nlm.nih.gov/pubmed/8450993

Barinaga M (1994) Neurotrophic factors enter the clinic. *Science*, 264: 772-774.

Beckman KB and Bruce NA (1998) The Free Radical Theory of Aging Matures. *Physiol. Rev.* 78: 547–581

Benowitz LI, Shashoua VE (1997) Localization of a brain protein metabolically linked with behavioral plasticity in the goldfish. *Brain Research*, 11 (2): 227-242.

Blurton-Jones M, Kitazawa M, Martinez-Coria H, Castello NA, Muller FJ, Loring JF, Yamasaki TR, Poon WW, Green KN, LaFerla FM (2009) Neural stem cells improve cognition via BDNF in a transgenic model of Alzheimer disease. *PNAS,* 106(32): 13594-9.

Boutajangout A, Quartermain D, Sigurdsson EM (2010) Immunotherapy Targeting Pathological Tau Prevents Cognitive Decline in a New Tangle Mouse Model. *Journal of Neuroscience*, 30(49): 16559 –16566.

Butterfield DA (2006) Amyloid β-Peptide(1-42), Oxidative Stress, and Alzheimer's Disease. *Free Radical Research*, 36(12): 1307-1313. http://informahealthcare.com/doi/pdf/10.1080/10715760210000498 90

Capsoni S, Giannotta S, Cattaneo A (2002) Nerve growth factor and galantamine amelio rate early signs of neurodegeneration in anti-nerve growth factor mice. *Proc Natl Acad Sci USA*, 99: 12432-12437. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC18754/

Chan PH and Kawase M (1998) Over-expression of SOD1 in transgenic rats protects vulnerable neurons against ischemic damage after cerebral ischemia and reperfusion. *J Neurosci*, 20: 8292-8299.

Chen G, Chen KS, Knox J, Inglis J, Bernard A, Martin SJ, Justice A, McConlogue L, Games D, Freedman SB, Morris Richard GM (2000) A learning deficit related to age and β-amyloid plaques in a mouse model of Alzheimer's disease. *Nature*, 408: 975-979.

Clark CM, Schneider JA, Bedell BJ, Beach TG, Bilker WB, Mintun MA, Pontecorvo MJ, Hefti F, Carpenter AP, Flitter ML, Krautkramer MJ, Kung HF, Coleman RE, Doraiswamy PM, Fleisher AS, Sabbagh MN, Sadowsky CH, Reiman EM, Zehntner SP, Skovronsky DM (2011) Use of florbetapir-PET for imaging β-amyloid pathology. *JAMA*, 305(3): 275-283. http://jama.ama-assn.org/content/305/3/275.short

Cramer PE, Cirrito JR, Wesson DW, Lee CY, Karlo JC, Zinn AE, Casali BT, Restivo JL, Goebel WD, James MJ, Brunden KR, Wilson DA, Landreth GE (2012) ApoE-directed therapeutics rapidly clear β-amyloid and reverse deficits in AD mouse models. *Science*, 335(6075): 1503-1506. http://www.sciencemag.org/content/335/6075/1503.full.pdf

Crews L, Masliah E (2010) Molecular mechanisms of neuro-degeneration in Alzheimer's disease. *Hum Mol Genet*, 19(R1): R12-20. http://www.ncbi.nlm.nih.gov/pubmed/20413653

Donahue M, and Lobdell G (2011) Role of SOD and Cathepsin-D in Alzheimer's Disease Aβ Cascade Models. WPI MQP Project, May, 2011.

Erickson KI, Prakash RS, Voss MW, Chaddock L, Heo S, McLaren M, Pence BD, Martin SA, Vieira VJ, Woods JA, McAuley E, Kramer AF (2010) Brain-Derived Neurotrophic Factor is Associated with Age-Related Decline in Hippocampal Volume. *J Neuroscience*, 30(15): 5368-5375.

Evans DA, Hebert LE, Beckett LA, Scherr PA, Albert MS, Chown MJ, Pilgrim DM, Taylor JO (1997) Education and other measures of socioeconomic status and risk of incident Alzheimer's disease in a defined population of older persons. *Archives of Neurology*, 54(11): 1399–1314. http://www.ncbi.nlm.nih.gov/pubmed/9362989

Evans DA, Bennett DA, Wilson RS, Bienias JL, Morris MC, Scherr PA, Hebert LE, Aggarwal N, Beckett LA, Joglekar R, Berry-Kravis E, Schneider J (2003) Incidence of Alzheimer's disease in a biracial urban community: Relation to apolipoprotein E allele status. *Archives of Neurology*, 60(2): 185–189. http://www.ncbi.nlm.nih.gov/pubmed/12580702

Fillenbaum GG, Heyman A, Huber MS, Woodbury MA, Leiss J, Schmader KE, Bohannon A, Trapp-Moen B (1998) The prevalence and 3-year incidence of dementia in older black and white community residents. *Journal of Clinical Epidemiology*, 51(7): 587–595. http://www.ncbi.nlm.nih.gov/pubmed/9674666

Fitzpatrick AL, Kuller LH, Ives DG, Lopez OL, Jagust W, Breitner JC, Jones B, Lyketsos C, Dulberg C (2004) Incidence and prevalence of dementia in the Cardiovascular Health Study. *Journal of the American Geriatrics Society*, 52(2): 195–204. http://www.ncbi.nlm.nih.gov/pubmed/14728627

Games D, Adams D, et al. (1995) Alzheimer-type neuropathology in transgenic mice over-expressing V717F β-amyloid precursor protein. *Nature*, 373: 523-527.

Gurland BJ, Wilder DE, Lantigua R, Stern Y, Chen J, Killeffer EH, Mayeux R (1999) Rates of dementia in three ethno-racial groups. *International Journal of Geriatric Psychiatry*, 14(6): 481–493. http://www.ncbi.nlm.nih.gov/pubmed/10398359

Hebert LE, Scherr PA, McCann JJ, Beckett LA, Evans DA (2001) Is the risk of developing Alzheimer's disease greater for women than for men? *American Journal of Epidemiology*; 153(2): 132–136. http://aje.oxfordjournals.org/content/153/2/132

Hebert LE, Scherr PA, Bienias JL, Bennett DA, Evans, DA (2003) Alzheimer's disease in the U.S. population: Prevalence estimates using the 2000 Census. *Archives of Neurology*, 60(8): 1119–1122. http://alzheimer.ucdavis.edu/outreach/wnews/pdf/1119.pdf

Hefti F (1997) Pharmacology of neurotrophic factors. *Annual Review of Pharmacology and Toxicology*, 37: 239-267.

Jellinger K, Schmidt R, & Windisch M (2002) Ageing and Dementia: Current and Future Concepts. Springer. Retrieved from http://books.google.com/books?hl=en&lr=&id=G831paIEzJMC&pgis=1

Jiang Q, Lee CY, Mandrekar S, Wilkinson B, Cramer P, Zelcer N, Mann K, Lamb B, Willson TM, Collins JL, Richardson JC, Smith JD, Comery TA, Riddell D, Holtzman DM, Tontonoz P, Landreth GE (2008) ApoE promotes the proteolytic degradation of A-beta. *Neuron*, 58(5): 681-93. http://www.ncbi.nlm.nih.gov/pubmed/18549781

Kadish I, Thibault O, Blalock EM, Chen KC, Gant JC, Porter NM, Landfield PW (2009) Hippocampal and cognitive aging across the lifespan: a bioenergetic shift precedes and increased cholesterol trafficking parallels memory impairment. *Journal of Neuroscience*, 29(6): 1805-1816. www.ncbi.nlm.nih.gov/pubmed/19211887

Kapoor V (2007) Mechanism of reversal of Alzheimer's disease Aβ-induced neuronal degeneration in cultured human SHSY cells using a neurotrophic ependymin mimetic. WPI Master's Thesis, July 2007.

Karran E, Mercken M, De Strooper B (2011) The amyloid cascade hypothesis for Alzheimer's disease: an appraisal for the development of therapeutics. *Nat Rev Drug Discov*, 10(9): 698-712.

Knopman DS, Boeve BF, Peterson RC (2003) Essentials of the proper diagnoses of mild cognitive impairment, dementia, and major subtypes of dementia. *Mayo Clin Proc*, 78: 1290-1308.

Kukull WA, Higdon R, Bowen JD, McCormick WC, Teri L, Schellenberg GD, van Belle G, Jolley L, Larson EB (2002) Dementia and Alzheimer's disease incidence: A prospective cohort study. *Archives of Neurology*, 59(11): 1737–1746. http://www.ncbi.nlm.nih.gov/pubmed/12433261

Levi-Montalcini R (1952) Effects of mouse tumor transplantation on the nervous system. *Ann. NY Acad. Sci.* 55:330-43

Levi-Montalcini R (1953) In-vivo and in vitro experiments on the effect of mouse sarcoma 180 and 37 on the sensory and sympathetic system of the chick embryo. *Proc. 14th Congr. Zool*., Copenhagen, p. 309

Levi-Montalcini R (1958) Chemical stimulation of nerve growth. In Chemical Basis of Development, ed. W. D. McElroy, B. Glass, ' pp. 64tHi4. Baltimore: John Hopkins Press

Levy YS, Gilgun-Sherki Y, Melamed E, Offen D (2005) Therapeautic Potential of Neurotrophic Factors in Neurodegenerative Diseases. *Biodrugs*, 19(2):97-127. http://www.ncbi.nlm.nih.gov/pubmed/15807629

Lovestone S and Reynolds CH (1997) The phosphorylation of tau: a critical stage in neurodevelopment and neurodegenerative processes. *Neuroscience* 78: 309–324.

Liu L, Drouet V, Wu JW, Witter MP, Small SC, Clelland C, Duff K (2012) Trans-synaptic spread of tau pathology in vivo. *PLoS One*. 7(2): e31302

Masters CL, Cappai R, Barnham KJ, Villemagne VL (2006) Molecular mechanisms for Alzheimer's disease: implications for neuroimaging and therapeutics. *J Neurochem*, 97(6): 1700-1725. http://www.ncbi.nlm.nih.gov/pubmed/16805778

McDowell I, Xi G, Lindsay J, Tierney M (2007) Mapping the connections between education and dementia. *Journal of Clinical and Experimental Neuropsychology*, 29(2): 127–141. http://www.ncbi.nlm.nih.gov/pubmed/17365248

Moran PM, Higgins LS, Cordell B, Moser PC (1995) Age-related learning deficits in transgenic mice expressing the 751-amino acid isoform of human 18-amyloid precursor protein. *PNAS*, 92: 5341-5345.

Morris R (1984) Development of a water-maze procedure for studying spatial learning in the rat. *J Neurosci Methods*, 11(1):47-60.

Murer MG, Yan Q, Raisman-Vozari R (2001) Brain-derived neurotrophic factor in the control human brain, and in Alzheimer's disease and Parkinson's. *Prog Neurobiol*, 63: 71–124.

Nagahara AH, Merrill DA, Coppola G, Tsukada S, Schroeder BE, Shaked GM, Wang L, Blesch A, Kim A, Conner JM, Rockenstein E, Chao MV, Koo EH, Geschwind D, Masliah E, Chiba AA, Tuszynski MH (2009) Neuroprotective effects of brain-derived neurotrophic factor in rodent and primate models of Alzheimer's disease. *Nat Med*, 15(3): 331-7.

Nalbantoglu J, Tirado-Santiago G, Lahsaini A, Poirier J, Goncalves O, Verge G, Momoli F, Welner SA, Massicotte G, Julien JP, Shapiro ML (1997) Impaired learning and LTP in mice expressing the carboxy terminus of the Alzheimer amyloid precursor protein. *Nature*, 387: 500–505.

O'Bryant SE, Hobson VL, Hall JR, Barber RC, Zhang S, Johnson L, Diaz-Arrastia R; Texas Alzheimer's Research Consortium (2011) Serum brain-derived neurotrophic factor levels are specifically associated with memory performance among Alzheimer's disease cases. *Dement Geriatr Cogn Disord*, 31(1): 31-36. http://www.ncbi.nlm.nih.gov/pubmed/21135555

Parikh S (2003) Ependymin peptide mimetics that assuage ischemic damage increase gene expression of the anti-oxidative enzymes SOD. WPI Master's Thesis, April, 2003.

Perl DP (2000) Neuropathology of Alzheimer's disease and related disorders. *Neurol. Clin*. 18: 847–864.

Plassman BL, Langa KM, Fisher GG, Heeringa SG, Weir DR, Ofstedal MB, Burke JR, Hurd MD, Potter GG, Rodgers WL, Steffens DC, Willis RJ, Wallace RB (2007) Prevalence of dementia in the United States: The Aging, Demographics, and Memory Study. *Neuroepidemiology*, 29(1–2): 125–132. http://www.ncbi.nlm.nih.gov/pubmed/17975326

Potter GG, Plassman BL, Burke JR, Kabeto MU, Langa KM, Llewellyn DJ, Rogers MA, Steffens DC (2009) Cognitive performance and informant reports in the diagnosis of cognitive impairment and dementia in African-Americans and whites. *Alzheimer's & Dementia*, 5(6): 445–453. http://www.ncbi.nlm.nih.gov/pubmed/19896583

Progress Report on Alzheimer's Disease: Translating new knowledge. (2009) U.S. Department of Health and Human Services, National Institutes of Health, National Institute of Aging. NIH Publication Number: 10-7500.

Rapoport M, Dawson HN, Binder LI, Vitek MP, Ferreira A (2002) Tau is essential to β-amyloid-induced neurotoxicity. *Proceedings of the National Academy of Sciences, USA*, 99: 6364-6369.

Rawal D (2010) Use of a neurotrophic factor mimetic to block amyloid toxicity in Alzheimer's disease models. WPI Thesis, 2010.

Robertson ED, Scearce-Levie K, Palop JJ, Yan F, Cheng IH, Wu T, Gerstein H, Yu GQ, and Mucke L (2007) Reducing endogenous tau ameliorates amyloid β-induced deficits in an Alzheimer's disease mouse model. *Science*, 316: 750-754.

Roe CM, Xiong C, Miller JP, Morris JC (2007) Education and Alzheimer's disease without dementia: Support for the cognitive reserve hypothesis. *Neurology*, 68(3): 223–228. http://www.ncbi.nlm.nih.gov/pubmed/17224578

Ronayne R (2008) Human ependymin-1 neurotrophic factor mimetic reduces tau phosphorylation and cellular apoptosis *in vitro* and *in vivo* in Alzheimer's disease models. WPI Master's Thesis, September, 2008.

Sanders L (2011) Memories Can't Wait: researchers rethink the role of amyloid in causing Alzheimer's. *ScienceNews*, 179(6): 24.

Saxton J, Morrow L, Eschman A, Archer G, Luther J, and Zuccolotto A (2009) Computer assessment of mild cognitive impairment. *Postgrad Med*, 121(2): 177–185.

Selkoe D. J. (2001) Alzheimer's disease: genes, proteins, and therapy. *Physiol. Rev*. 81:741–766.

Shashoua VE, Adams D, Boyer-Boiteau A (2001) CMX-8933, a peptide fragment of the glycoprotein ependymin, promotes activation of AP-1 transcription factor in mouse neuroblastoma and rat cortical cell cultures. *Neuroscience Letters*, 312: 103-107.

Shashoua VE, Adams DS, Boyer-Boiteau A, Cornell-Bell A, Li F, Fisher M (2003) Neuroprotective effects of a new synthetic peptide, CMX-9236, in in vitro and in vivo models of cerebral ischemia. *Brain Research,* 963: 214-223.

Shashoua VE, Adams DS, Volodina NV, Li H (2004) New synthetic peptides can enhance gene expression of key antioxidant defense enzymes in vitro and in vivo. *Brain Research,* 1024: 34-43.

Shen L, Figurov A, Lu B (1997) Recent progress in studies of neurotrophic factors and their clinical applications. *Journal of Molecular Medicine*, 75: 637-644.

Sheng H, Bart RD, Oury TD, Pearlstein RD, Crapo JD, Warner DS (1999) Mice over-expressing extracellular superoxide dismutase have increased resistance to focal cerebral ischemia. *Neuroscience,* 88: 185-191.

Shriver M (2010) The Shriver Report: A Woman's Nation Takes on Alzheimer's. Alzheimer's Association. http://www.alz.org/shriverreport/index.html

Smith MA, Rottkamp CA, Nunomura A, Raina AK, Perry G (2000) Oxidative stress in Alzheimer's disease. *Biochimica et Biophysica Acta*, 1502: 139-144.

Stern Y, Gurland B, Tatemichi TK, Tang MX, Wilder D, Mayeux R (1994) Influence of education and occupation on the incidence of Alzheimer's disease. *Journal of the American Medical Association*, 271(13): 1004–1010. http://www.ncbi.nlm.nih.gov/pubmed/8139057

Stern Y (2006) Cognitive reserve and Alzheimer disease. *Alzheimer Disease & Associated Disorders*, 20(2): 112–117. http://journals.lww.com/alzheimerjournal/Abstract/2006/04000/Cognitive_Reserve_and_Alzheimer_Disease.6.aspx

Stovall K (2006) Partial restoration of cell survival by a human ependymin mimetic in an *in vitro* Alzheimer's disease model. WPI Master's Thesis, August, 2006.

Tuszynski M, Gage F (1994) Neurotrophic factors and diseases and the nervous system. *Annals of Neurology*, 35: S9-S12.

Venarucci D, Venarucci V, Vallese A, Battila L, Casado A, De la Torre R, Lopez-Fernandez ME (1999) Free radicals: important cause of pathologies related to ageing. *Panminerva Medicine,* 4: 335-339.

Yaffe K, Weston A, Graff-Radford NR, Satterfield S, Simonsick EM, Younkin SG, Younkin LH, Kuller L, Ayonayon HN, Ding J, Harris TB (2011) Association of plasma β-amyloid level and cognitive reserve with subsequent cognitive decline. *JAMA*, 305(3): 261-266. http://jama.ama-assn.org/content/305/3/261.short

Yan SD, Chen X., Fu J, Chen M, Zhu H, Roher A, Slattery T, Zhao L, Nagazhima M, Morser J, Mighelo A, Nawroth P, Stern D, Schmidt AM (1996) RAGE and amyloid-β peptide neurotoxicity in Alzheimer's disease. *Nature,* 382: 685-691.

Yao J, Irwin RW, Zhao L, Nilson J, Hamilton RT, Brinton RD (2009) Mitochondrial bioenergetic deficit precedes Alzheimer's pathology in female mouse model of Alzheimer's disease. *Proceedings of the National Academy of Sciences,* 106(34): 14670-14675.

Zuccato C, Cattaneo E (2009) Brain-derived neurotrophic factor in neurodegenerative diseases. *Nature Reviews Neurology,* 5: 311-322.

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